IDENTIFICATION AND CHARACTERIZATION OF GENES ASSOCIATED WITH THE HORMONAL PROGRESSION OF PROSTATE CANCER

by

STEVEN NORMAN QUAYLE
B.Sc., The University of British Columbia, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
(PATHOLOGY AND LABORATORY MEDICINE)

THE UNIVERSITY OF BRITISH COLUMBIA
August 2006

© Steven Norman Quayle, 2006
ABSTRACT

Prostate cancer is the most frequently diagnosed cancer as well as the second leading cause of cancer deaths among American men. Androgen deprivation therapy for patients with advanced prostate cancer inevitably fails as the disease progresses to the terminal, hormone-refractory stage. At the present time no effective therapies can be offered to patients in this stage of the disease. Currently, the molecular alterations that contribute to the development of hormone-refractory disease are poorly understood. Here, suppression subtractive hybridization was performed to identify genes of interest in a model of progression of prostate cancer to androgen independence. Subtractive hybridization identified a number of novel transcripts that had previously not been described. From this work it was hypothesized that (1) the novel transcripts identified may prove informative in the clinical management of prostate cancer, and (2) that the known genes identified may function in the progression of prostate cancer.

One of these novel transcripts was shown to encode a truncated splice variant of the transmembrane protein TMEFF2. TMEFF2 is specifically expressed in the prostate and the brain, and other groups have developed antibodies targeting TMEFF2 for the treatment of metastatic prostate cancer. The isoform described here, TMEFF2-S, encodes a protein secreted by prostate cancer cells. Being a prostate-specific, secreted protein, TMEFF2-S may prove informative in predicting patient outcome. Additionally, the secretion of TMEFF2-S may influence the effectiveness of immunotherapy approaches targeting TMEFF2.

Subtractive hybridization, together with other high-throughput screening techniques, also led to the examination of the potential functions of the 14-3-3 family of
proteins in the progression of prostate cancer. These experiments demonstrated that 14-3-3 sigma specifically increased the activity of the androgen receptor in the absence of androgens. Thus, this finding may have implications for patients undergoing androgen deprivation therapy. Further studies will be required to determine if 14-3-3 sigma is active in a subset of patients with hormone refractory prostate cancer. If so, its role in activating the androgen receptor may potentially be targeted for the development of novel therapeutics for the treatment of hormone-refractory disease.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xii</td>
</tr>
<tr>
<td>Co-Authorship Statement</td>
<td>xiv</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Natural history of prostate cancer</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Diagnosis of prostate cancer</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Treatment of prostate cancer</td>
<td>4</td>
</tr>
<tr>
<td>1.1.3 Recurrence of prostate cancer</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Androgen receptor</td>
<td>7</td>
</tr>
<tr>
<td>1.3 Mechanisms of androgen independent growth</td>
<td>11</td>
</tr>
<tr>
<td>1.4 Models for studying androgen-independent disease</td>
<td>16</td>
</tr>
<tr>
<td>1.5 Differential gene expression</td>
<td>19</td>
</tr>
<tr>
<td>1.5.1 Gene expression changes in prostate cancer</td>
<td>19</td>
</tr>
<tr>
<td>1.5.2 Techniques for transcriptional profiling</td>
<td>21</td>
</tr>
<tr>
<td>1.6 Suppression subtractive hybridization</td>
<td>23</td>
</tr>
<tr>
<td>1.7 Summary and research objectives</td>
<td>26</td>
</tr>
<tr>
<td>1.7.1 Hypothesis</td>
<td>27</td>
</tr>
<tr>
<td>1.7.2 Specific aims</td>
<td>27</td>
</tr>
<tr>
<td>1.7.3 Thesis overview</td>
<td>28</td>
</tr>
<tr>
<td>1.8 References</td>
<td>30</td>
</tr>
<tr>
<td>2 Novel expressed sequences identified in a model of androgen-independent prostate cancer</td>
<td>50</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>50</td>
</tr>
<tr>
<td>2.2 Materials and methods</td>
<td>52</td>
</tr>
<tr>
<td>2.2.1 Cell culture</td>
<td>52</td>
</tr>
<tr>
<td>2.2.2 LNCaP Hollow fibre model</td>
<td>52</td>
</tr>
<tr>
<td>2.2.3 Suppression subtractive hybridization</td>
<td>54</td>
</tr>
<tr>
<td>2.2.4 RT-PCR</td>
<td>55</td>
</tr>
</tbody>
</table>
2.3 Results

2.3.1 Subtractive hybridization

2.3.2 Novel transcripts were represented in the subtracted cDNA libraries

2.3.3 Characterization of novel clone sequences

2.3.4 Analysis of SAGE tags in the novel clones

2.3.5 The novel subtracted clones are expressed by a variety of cell types

2.3.6 Two of the novel clones are part of larger untranslated regions

2.4 Discussion

2.5 References

3 A truncated isoform of TMEFF2 encodes a secreted protein in prostate cancer cells

3.1 Introduction

3.2 Materials and methods

3.2.1 Cell culture and transfection

3.2.2 RT-PCR

3.2.3 Plasmids

3.2.4 Western blot analysis

3.2.4 Fluorescent microscopy

3.3 Results

3.3.1 A novel splice variant is transcribed from the TMEFF2 locus

3.3.2 TMEFF2-S was expressed in normal human tissues and produced a truncated protein

3.3.3 The truncated isoform of TMEFF2 produced a secreted protein

3.4 Discussion

3.5 References

4 14-3-3 sigma increases the transcriptional activity of the androgen receptor in the absence of androgens

4.1 Introduction

4.2 Materials and methods

4.2.1 Cell culture

4.2.2 Plasmids

4.2.3 Transfection and luciferase assay

4.2.4 AR siRNA

4.2.5 Western blot analysis

4.2.6 Co-immunoprecipitation

4.3 Results

4.3.1 14-3-3 sigma specifically enhanced AR transcriptional activity

4.3.2 14-3-3 sigma enhanced AR activity in a dose-dependent manner in the absence of androgens

4.3.3 14-3-3 sigma increased the sensitivity of AR to low levels of androgens
4.3.4 Induction of PSA reporters by 14-3-3 sigma was dependent on AR..... 112
4.3.5 14-3-3 sigma did not alter AR protein levels........................................ 117
4.4 Discussion................................................................................................. 119
4.5 References............................................................................................... 124

5 Concluding Chapter.................................................................................... 130

5.1 Expression profiling of novel clones.......................................................... 130
  5.1.1 Custom cDNA microarray...................................................................... 130
  5.1.2 Low-density TaqMan® array................................................................. 132
5.2 Summary and future directions................................................................. 133
  5.2.1 Novel transcripts.................................................................................. 134
  5.2.2 TMEFF2-S......................................................................................... 137
  5.2.3 14-3-3 sigma..................................................................................... 139
5.3 Conclusions............................................................................................... 142
5.4 References............................................................................................... 144

6 Appendix..................................................................................................... 150

6.1 Animal Care Certificate........................................................................... 150
6.2 Biohazard Approval Certificate............................................................... 151
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Primers used for RT-PCR</td>
<td>56</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Summary of sequenced clones</td>
<td>59</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Sequence characteristics of unannotated clones</td>
<td>62</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Sequence characteristics of clones matching previously identified ESTs</td>
<td>63</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Sequence characteristics of clones matching previously annotated cDNAs</td>
<td>64</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Page No.

Figure 1.1  Mechanism of ligand-dependent activation of the androgen receptor .................................................. 9

Figure 1.2  Proposed mechanisms of androgen-independent growth and survival of prostate cancer .................................................................................................................. 12

Figure 1.3  Schematic diagram of suppression subtractive hybridization protocol .............................................. 25

Figure 2.1  Distribution of sequence matches in the suppression subtractive hybridization clone set .......................................................... 60

Figure 2.2  The novel subtracted clones were expressed in a variety of human tissues ........................................... 68

Figure 2.3  Two of the novel clones were part of transcripts containing a neighbouring SAGE tag .............................................. 71

Figure 3.1  A novel EST is part of a larger transcript derived from the TMEFF2 locus .................................................. 90

Figure 3.2  The novel splice variant of TMEFF2 is expressed in normal tissues ................................................. 92

Figure 3.3  The novel splice variant of TMEFF2 produced a truncated protein .................................................... 93

Figure 3.4  The truncated variant of TMEFF2 is not localized in the plasma membrane but is secreted by transfected cells.......................................................... 95

Figure 4.1  The transcriptional activity of the AR was activated specifically by 14-3-3 sigma .......................................................... 110

Figure 4.2  Increasing amounts of 14-3-3 sigma increased the amount of AR activity in the absence of androgens .......................................................... 113

Figure 4.3  14-3-3 sigma sensitized the AR to low levels of androgen .......................................................... 114
Figure 4.4  Activation of the ARR3-tk-luciferase reporter construct by 14-3-3 sigma was dependent on the AR. 115

Figure 4.5  14-3-3 sigma did not interact with or alter the amount of AR protein in the cells. 118
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen response element</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal exam</td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HRPC</td>
<td>Hormone-refractory prostate cancer</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph node-derived carcinoma of the prostate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor co-repressor</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
</tbody>
</table>
RT-PCR Reverse-transcriptase polymerase chain reaction
SAGE Serial analysis of gene expression
SRC-1 Steroid receptor co-activator 1
SSH Suppression subtractive hybridization
tk Thymidine kinase
TMEFF2 Transmembrane protein with EGF-like and two follistatin-like domains 2
UTR Untranslated region
ACKNOWLEDGEMENTS

A number of people at the BC Cancer Research Centre have helped me with the experiments described in this thesis. Nasrin Mawji and Dr Rob Snoek provided me with seemingly endless advice and assistance in the design and execution of a number of experiments. Heidi Hare provided me with excellent technical assistance. Dorothy Hwang and Jean Wang maintained the LNCaP hollow fibre model and monitored the experiments. Dr Allen Delaney and Scott Zuyderduyn provided bioinformatics support and performed comparisons to various SAGE libraries. And Dr Steven Jones provided invaluable assistance and knowledge of the world of bioinformatics. I was also fortunate to have the help of three undergraduate students – Stephanie Warner, Sherman Tung, and Michelle Joe.

A number of people have also provided helpful discussions and advice on everything from experimental design to converting computer files. In no particular order, they are: Tammy Romanuik, Dr Katie Meehan, Dr Brad Hoffman, Elizabeth Hajen, Amara Garcia, Brad Coe, Angela Beckett, Dr James Goldie, and Dr Nicholas Bruchovsky. Other members of the Sadar laboratory have also provided helpful suggestions and comments: Dr Viola Akopian, Dr Tom Kim, Natasha Mendelev, Hans Adomat, Natalie Blaszczyk, Dr Richard Sobel, Gang Wang, Helen Chiu, Teresa Tam, and Dr Barbara Comuzzi. Dr Wan Lam allowed me to use his microarray scanner and provided much encouragement. A number of administrative assistants have also helped me over the years, including Susan Chou, Penny Woo, Louise Clarke, and Mary Flores.
I was financially supported by a number of agencies. Scholarships were received from the Canadian Institutes of Health Research, the Michael Smith Foundation for Health Research, the University of British Columbia, and the Department of Pathology and Laboratory Medicine. Travel awards were provided by the Society for Basic Urologic Research, Keystone Symposia, and Albert B. and Mary Steiner.

I am of course indebted to my supervisor, Dr Marianne Sadar. She provided me with a rich research environment and financial support for both my stipend and my research (through a Centre of Excellence for Prostate Cancer Research grant from Health Canada). And she was always generous with her time while we discussed experiments, conferences, and holidays.

On a personal note, I have to acknowledge my girlfriend, Lynn Mar, for all that she has given me during my training: endless advice on cloning, critical analysis of my experiments, and constant support when we were exhausted and it seemed like we would never be finished. And finally, this thesis would not have been possible without the unlimited support and encouragement of my family.

Thank you all.
CO-AUTHORSHIP STATEMENT

The experiments described in this thesis were conceived, designed, conducted, and analyzed by myself, Steven Quayle, and Dr Marianne Sadar. However, a number of people contributed to the work described in Chapter 2. Dr Steven Jones and Dr Marco Marra helped conceive and design the experiments. The experiments were performed by Steven Quayle, Heidi Hare, Dr Allen Delaney, Dorothy Hwang, and Jacqueline Schein. Steven Quayle analyzed the data. Finally, Steven Quayle prepared the manuscripts and this thesis.
1 INTRODUCTION

1.1 Natural history of prostate cancer

The prostate is a small secretory organ surrounding the urethra at the base of the bladder. Its normal function is to secrete factors into the semen to increase survival of the sperm. The prostate is divided into three histologic regions: the transition zone, the central zone, and the peripheral zone. The transition zone is the smallest (5% of prostate volume), followed by the central zone (25% of prostate volume), with the peripheral zone being the largest (70% of prostate volume) [1,2]. The entire prostate is surrounded by a "capsule" of fibromuscular stromal cells [3]. Androgens regulate the differentiation, proliferation, secretion of proteins, as well as various biosynthetic pathways in the normal prostate. In fact, the prostate is an androgen dependent tissue and the removal of androgens results in apoptotic cell death [4,5]. It is this dependence on androgens that forms the basis for the treatment of advanced prostate cancer with androgen deprivation therapy (ADT), which will be discussed in more detail below. Androgens exert their effects on the prostate through direct binding to the androgen receptor (AR), a transcription factor that is a member of the nuclear receptor superfamily [6].

1.1.1 Diagnosis of prostate cancer

Carcinoma of the prostate is the most frequently diagnosed cancer as well as the second leading cause of cancer death among American men [7]. The majority of prostate cancers are located in the peripheral zone [2,8]. Since the peripheral zone lies adjacent to the rectum, it is possible to detect abnormal prostate morphology, indicative of
hyperplastic growth, through a digital rectal exam (DRE). The DRE is most useful in diagnosing peripheral zone cancers as cancerous foci located in the transition and central zones are less likely to be detected by this technique.

The DRE is one of three tests used to diagnose prostate cancer. The second means of diagnosis relies on the measurement of serum levels of the prostate-specific antigen (PSA) protein. PSA is a 34 kDa serine protease normally expressed and secreted into the semen by the luminal epithelial cells of the prostate [9,10]. It was first purified in 1979 and was later shown to be important for the liquefaction of ejaculated semen [9-11].

However, development of a neoplastic growth in the prostate, or invasion of a prostatic carcinoma through the capsule, can result in elevated circulating levels of PSA in the bloodstream. Serum PSA levels can be accurately measured in these patients through the use of various techniques, including radioimmunoassay. Numerous studies indicate PSA expression is generally restricted to the prostate and that the level of serum PSA correlates well with the palpable stages of prostate cancer, leading to its use as a diagnostic marker in screening for prostate cancer [12-14]. The final, and most definitive, method of diagnosing prostate cancer remains the pathologic identification of invasive carcinoma after biopsy.

Despite the value of PSA as a marker of prostate cancer there is currently a great deal of controversy in the field regarding its usefulness for screening programs. There is a growing body of literature suggesting that prostate cancer is present in men with no clinical symptoms of the disease and normal diagnostic parameters, including PSA levels. A classic study by Sakr et al. [15] performed pathological analyses on the prostates of a
large number of men who died of causes other than prostate cancer and who had no clinical symptoms of prostate cancer. This study found that up to 80% of men from 70 – 80 years of age had precancerous lesions in their prostates [15]. Additionally, the Prostate Cancer Prevention Trial examined a large population of men and found that amongst men with “normal” PSA and DRE tests, approximately 15% could be diagnosed with prostate cancer based on a biopsy [16]. Results such as these have led some to conclude that prostate cancer is currently being underdiagnosed.

Over the last decade there has been a steady decrease in the number of patients diagnosed with “high-risk” disease and a concomitant increase in diagnosis of “low-risk” disease, suggesting that prostate cancer is now being detected at earlier stages [17]. It has been recommended that lowering the PSA threshold may aid in identifying more cases of prostate cancer in younger men [18]. However, as cutoff values for “abnormal” PSA are lowered the sensitivity and specificity of the PSA test will also decrease. This means that more men will be referred for prostate biopsies, but a lower percentage of those men will be diagnosed with prostate cancer. In fact, a recent study has called into question the diagnostic value of serum PSA measurements below 22 ng/mL [19]. Further complicating the issue is the fluctuations in PSA level that are known to occur with time, and particularly with disturbances of the prostate [13,20]. New markers are thus needed to classify patients into high- and low-risk categories. Regardless of these controversies, prostate cancer is diagnosed in a large number of men each year [7]. The question then is how they should be treated.
1.1.2 Treatment of prostate cancer

Upon diagnosis of prostate cancer by biopsy, these patients are typically offered a number of treatment options (for recent, in depth reviews, see [21-24]). The first, and most common, is radical prostatectomy where the entire prostate and the seminal vesicles are removed by surgery. The second is external beam radiation therapy targeting the prostate. More recent advances have led to the development of brachytherapy for prostate cancer where radioactive pellets are implanted directly into the prostate where they steadily emit a low level of radiation. Each of these interventions is undertaken with curative intent. However, given the generally slow progression of prostate cancer [25,26], it is not always advantageous to attempt a full cure. For example, active surveillance, or watchful waiting, may be offered to either those at lowest risk of disease recurrence or those who would suffer excessive morbidity from the other treatment options. The intent of this approach is obviously not curative, but simply to maximize quality of life while still being prepared to actively treat the patient should their situation worsen. Finally, androgen deprivation may be offered prior to or in conjunction with all of the above treatments.

Two studies have recently looked at long term outcomes in patients whose cancers were not treated initially but were instead monitored by watchful waiting [25,26]. Both studies suggest that aggressive treatment is appropriate for patients whose cancers are poorly differentiated at diagnosis, but not for patients with low-grade disease. Encouragingly, a phase III study comparing radical prostatectomy to watchful waiting in patients with intermediate-risk disease found that surgery did result in decreased disease recurrence and decreased likelihood of death due to prostate cancer [27]. So while
patients diagnosed with more aggressive disease have a poorer outcome on watchful waiting, active treatment is able to extend their lives.

Unfortunately, it is not always easy to distinguish those patients who are likely to die from their disease, and thus require treatment, from those whose disease is likely to remain clinically insignificant. The result is that many men with intermediate-grade disease may be being over-treated [21,23]. Stamey et al. [28] found that the percentage of high grade cancer and the total cancer volume at diagnosis were the best predictors of biochemical relapse after radical prostatectomy. Other studies have tried to obtain better prognostic information through the examination of PSA kinetics in these patients. For example, a rate of rise of PSA, or PSA velocity, at diagnosis of greater than 2.0 ng/mL/year correlated with an increased risk of dying from prostate cancer after radical prostatectomy [29]. However, the classical indicators of progression – PSA level, grade, and clinical tumour stage at diagnosis – were also predictive of increased risk of death in this study [29]. New biomarkers capable of distinguishing clinically significant from clinically indolent disease need to be identified to aid in making these treatment decisions.

While the value of PSA as a diagnostic tool has been questioned, monitoring PSA levels post-diagnosis is an informative method of following the patient’s response to therapy. Initial curative therapy typically results in an undetectable level of PSA, but the appearance of increased serum PSA after therapy, referred to as biochemical failure, is associated with the development of distant metastases and an overall poor prognosis [13,30,31]. In fact, in men treated by radical prostatectomy alone, Pound et al. [32] found that disease recurrence was only observed in men demonstrating biochemical
relapse. In this study group of 1,997 men, 15% exhibited PSA recurrence in a median time of 2.3 years post-surgery [32]. After biochemical failure, 34% of patients developed clinical metastases within a median time of 5 years [32]. Thus, biochemical relapse is a signal to begin more active screening for metastases. From the appearance of clinical metastases, the median time to death due to prostate cancer was 5 years. However, 82% of the study group were metastasis free 15 years after surgery [32].

1.1.3 Recurrence of prostate cancer

Recurrence of the disease at distant metastatic sites necessitates a systemic therapy; ADT is the only therapeutic approach shown to result in regression of the disease. Chemical, via luteinizing hormone releasing hormone agonists such as goserelin, or surgical castration, via orchiectomy, results in decreased serum testosterone levels, decreased serum PSA levels, and regression of the tumour [4,31,33,34]. Additionally, non-steroidal antiandrogens, such as bicalutamide, can be used to block the activity of the AR directly, but these therapies are costly and it is not yet clear if bicalutamide alone increases overall survival [35,36]. Finally, these therapies have been tested together to achieve greater inhibition of AR activity, but again this treatment did not lead to a significant increase in survival [34,35,37,38]. Unfortunately, ADT is not curative, and even with therapy the median survival of these patients is only about 2.5 years [37].

The optimal timing to begin ADT is still under debate (for recent reviews, see [24,35]). Often, ADT would be initiated at the point of biochemical recurrence after initial curative therapy or in cases where metastatic disease is already present at the time
of diagnosis. Increasingly, though, ADT is being offered soon after primary curative therapy (i.e. surgery), and before biochemical relapse. Finally, phase III trials are now underway to assess the effectiveness of intermittent androgen deprivation in delaying the eventual development of hormone refractory disease (reviewed by [39]). Regardless of when ADT is started during disease progression, the development of hormone-refractory prostate cancer (HRPC) leaves patients with few effective treatment options.

Hormone-refractory, or androgen-independent, prostate cancer is that stage of disease after the failure of ADT, usually characterized by a rising serum PSA while still on therapy. The prognosis for patients at this stage of the disease is poor; the median survival is approximately 16 months [40-42]. In 2004, two studies showed an increase in overall survival among patients with HRPC who were treated with the chemotherapeutic drug docetaxel [41,42]. These were the first trials to demonstrate a therapy could increase survival in this group of patients, even though it was only a two month increase.

There is currently a significant lack of understanding of the molecular mechanisms leading to the development of hormone-refractory prostate cancer. Greater comprehension of these mechanisms may aid in the creation of novel therapies for these patients. Additionally, new biomarkers are needed to identify those patients who may benefit from aggressive therapy and those who should be offered palliative approaches to increase their quality of life.

1.2 Androgen receptor

As discussed above, the prostate is an androgen dependent organ and thus requires androgens for normal growth and differentiation. The effects of androgens are
mediated through binding of the hormone to its receptor, the AR. Unliganded AR is predominantly found in the cytoplasm, but upon binding ligand, such as dihydrotestosterone (DHT) or testosterone, the AR forms homodimers and translocates to the nucleus where it acts as a transcription factor for androgen regulated genes (Fig. 1.1) [43,44]. Nuclear translocation begins within 5-10 minutes after treatment of the cells with androgens, and is nearly complete within 60 minutes of treatment [44-46]. Once in the nucleus the AR binds androgen response elements (ARE) in the promoter and enhancer regions of its target genes and regulates transcriptional activity in association with co-activator proteins, such as steroid receptor co-activator 1 (SRC-1) [47,48], and co-repressor proteins, such as nuclear receptor co-repressor (NCoR) [49,50]. Examples of well-characterized androgen-regulated genes include PSA and ornithine decarboxylase [51,52].

The primary structure of the AR consists of four functional domains: an N-terminal transactivating domain, a DNA-binding domain, a hinge region, and a C-terminal ligand binding domain (LBD; reviewed in [53]). The amino (N)-terminal region contains the activating function (AF)-1 region (amino acid residues 110-379) that is primarily responsible for the transcription activating activity of the AR. This region also shows the least amount of homology across the nuclear receptor family. The DNA-binding domain contains two zinc-finger motifs (residues 559-614) that determine the specificity of interaction of the receptor with AREs and are also involved in the protein-protein interaction with the second AR molecule. Translocation of the AR to the nucleus is predominantly mediated by a ligand-dependent classical nuclear localization signal located within the DNA binding domain and hinge region of the AR (residues 617-633).
Figure 1.1 – Mechanism of ligand-dependent activation of the androgen receptor. In the absence of ligand AR is bound to chaperone proteins in the cytoplasm. Binding of ligands, such as DHT, leads to dissociation from its chaperone complex, phosphorylation, dimerization, and translocation to the nucleus. There it regulates the expression of target genes in conjunction with co-activators such as SRC-1. See text for details. AR, androgen receptor; DHT, dihydrotestosterone; hsp, heat shock protein; SRC-1, steroid receptor coactivator-1; RNA pol II, RNA polymerase complex. Adapted from [54].
The carboxyl (C)-terminal domain, extending from residues 676-919, is the region responsible for binding ligand. While ligand binding is predominantly mediated by a large hydrophobic cavity known as the ligand binding pocket (approximately residues 735-787 and 855-865), deletion of small regions of the ligand binding domain tends to result in greatly decreased ligand binding efficiency [57].

Steroid receptors that are not bound to ligand do not exist as free cytosolic monomers. Instead, they are found in large heteromeric complexes with heat shock proteins (Hsp) and other members of the cellular chaperone machinery (reviewed in [58]). Each AR molecule is bound to Hsp70 and Hsp40, which results in the recruitment of Hsp90 to the complex. Various other cochaperones are required for the formation of this complex, but these three factors are obligately associated with unliganded steroid receptors. Complexing with heat shock proteins may increase the stability of the unliganded receptor’s conformation so it can bind ligand. Alternatively, these chaperones may act to inhibit indiscriminate activation of the unliganded receptor by decreasing the efficiency of DNA binding and dimerization. Binding of agonists, such as DHT, by the receptor results in a conformational change in the AR, followed by dissociation of the multiprotein complex and trafficking of the activated AR through the cytoplasm and into the nucleus [59-61]. On the other hand, binding of antagonists, such as bicalutamide, prevents dissociation of the chaperone proteins and may inhibit the initial conformational change, but still induces nuclear import of the receptor [60,62,63].

Once AR has been activated and enters the nucleus it binds to AREs present in the enhancer regions of androgen-regulated genes. The AR binds as a homodimer to the consensus inverted repeat ARE, GGTACAnnnTGTTCT [64]. While numerous
androgen-regulated genes have been found, AR binding to AREs in the PSA promoter has been the most extensively studied [48,50,65]. Expression of the PSA gene is known to be androgen-regulated, and thus, monitoring PSA transcript levels is a simple method of assaying for transcriptional activation of the AR, in addition to its role in monitoring therapeutic response in prostate cancer patients.

1.3 Mechanisms of androgen independent growth

A growing body of evidence indicates the AR is transcriptionally active in androgen-independent prostate cancer cells. Androgen-independent disease is marked by increased expression of the AR and re-expression of AR-regulated genes, such as PSA, after recurrence of an androgen-deprived tumour [66,67]. In fact, the work of Chen et al. [68] suggested that the AR was the only gene consistently upregulated amongst several models of androgen independent disease, and increased expression of AR was also seen in clinical samples of HRPC [67,69]. Several models have been proposed to explain how normally androgen-dependent prostate cells are able to proliferate in the absence of androgens (Fig. 1.2) [70,71].

One such model postulates that the AR is activated in a ligand-independent manner through cross-talk with other signal transduction pathways (Fig. 1.2A). Evidence has been presented that non-androgenic compounds and growth factors, such as forskolin, insulin-like growth factor-I and interleukin (IL)-6, are able to induce nuclear import and the transcription activation function of AR even in the absence of androgens [72-76]. This effect may be dependent on phosphorylation of additional sites within the AR or in receptor-associated proteins such as the co-activator SRC-1 [74,76,77]. Phosphorylation
Figure 1.2 – Proposed mechanisms of androgen-independent growth and survival of prostate cancer. (A) Signalling events downstream of a GF receptor result in transcriptional activation of the AR in the absence of ligand. (B) Mutation or amplification of the AR enable the receptor to become activated after binding a wider range of ligands. (C) Increased expression of Bcl2 and/or loss of PTEN lead to increased survival signals. See text for details. AR, androgen receptor; GF, growth factor; coA, co-activator; hsp, heat shock protein; SRC-1, steroid receptor co-activator-1; RNA pol II, RNA polymerase complex; E2, estradiol. Adapted from [70].
of serine-650 in AR was also observed in response to both androgens and forskolin [78,79].

A number of in vitro experiments have shown that forskolin is able to induce ligand-independent activation of the AR [73,74]. This effect occurs through an increase in intracellular cyclic AMP levels, leading to activation of protein kinase A and phosphorylation of its downstream targets. The result is the production of endogenous PSA mRNA dependent on the activity of the AR [74]. Forskolin was also shown to increase the transcriptional activation function of the N-terminal domain of AR as well as increase the affinity of AR for binding to AREs [74].

There is also evidence suggesting ligand-independent activation of the AR may occur in vivo. An increase in serum levels of IL-6 has been associated with the progression of prostate cancer [80-82]. Nakashima et al. [80] demonstrated that serum IL-6 levels were significantly correlated with clinical stage in 74 patients at various stages of disease progression. Another group [82] found that IL-6 was elevated in androgen-independent disease relative to normal controls, benign prostatic hyperplasia, and localized and recurrent disease. Binding of IL-6 to its receptor results in downstream signalling through the Janus kinase family of proteins as well as through the mitogen-activated protein kinase (MAPK) cascade. MAPK, a kinase that regulates SRC-1 activity through phosphorylation, has been suggested to play a role in androgen-independent prostate cancer [83]. It has also been shown that treatment with IL-6 alone is sufficient to induce transcription of several androgen responsive promoters as well as the endogenous PSA gene in vitro [75,76,84,85]. Additionally, IL-6 treatment has been shown to increase the proportion of AR in the nucleus as well as increase the binding of AR to
androgen response elements [85]. Finally, the co-activator p300 was recently suggested to activate the expression of PSA independent of the AR in LNCaP cells after long term treatment with IL-6 [86].

A second mechanism by which the AR may become active in the absence of androgens is through increased promiscuity of the receptor (Fig. 1.2B). Increases in the copy number of AR and the amount of AR protein have been correlated with development of HRPC in 20-30% of patients [87,88]. Increased AR expression was also shown to sensitize prostate cancer cells to lower levels of androgens as well as the antiandrogen bicalutamide [68]. A number of mutations have also been identified in the AR of prostate cancer cell lines and patients that allow the receptor to be activated after binding a wider range of ligands, as well as antiandrogens [62,89-93]. However, approximately 10% of patients with HRPC had AR mutations, though this may be dependent on the length of therapy, and there was no correlation between the presence of AR mutations and overall survival [92,94]. It is therefore likely that the mechanism of androgen independence in approximately 60-70% of HRPC patients is not dependent on amplification or mutation of the AR. Additionally, these studies demonstrate that the AR is expressed in a large proportion of HRPC patients, suggesting that the loss-of-function of AR is not the predominant mechanism of androgen-independent growth.

The survival of prostate cancer cells after ADT indicates these cells are proliferating in the absence of androgens, perhaps due to the mechanisms discussed above. However, it is also possible that these surviving cancer cells may not undergo the typical apoptotic response to androgen withdrawal (Fig. 1.2C). These alternative theories are not mutually exclusive, and in fact are likely complementary. Consistent with this,
expression of the anti-apoptotic gene BCL2 has been found to increase with progression to HRPC [95]. Additionally, loss of the tumour suppressor PTEN has been correlated with prostate cancer progression and has been shown to increase cell survival through activation of Akt [96-98]. Mechanisms such as these can be utilized by the tumour to overcome the pro-apoptotic signals resulting from the removal of androgens, allowing the cancer cells to survive and grow in an androgen-independent manner.

The AR is expressed in the nucleus of a high proportion of HRPC tumours. This finding suggests the growth of the tumour may be promoted by AR activated in the absence of androgens. However, these cells may have additional abnormalities that also help them to survive ADT therapy. It is likely that there are multiple mechanisms leading to the androgen-independent growth of prostate cancer cells, and no single mechanism is sufficient to explain the growth of all cases of HRPC.

1.4 Models for studying androgen-independent disease

Given the difficulty of obtaining androgen-independent tissue from HRPC patients, a number of groups have attempted to develop in vitro and in vivo models that recapitulate the hormonal progression of prostate cancer.

The most frequently used prostate cancer cell line is the lymph node-derived carcinoma of the prostate (LNCaP) line originally derived from a lymph node metastasis of a human prostate cancer. This cell line expresses a functional AR, is responsive to androgens such as DHT, and expresses the prostatic marker prostatic acid phosphatase (PAP) [99,100]. LNCaP cells also express PSA mRNA and protein, which can be used as a natural androgen-responsive reporter [51,101]. The AR expressed by these cells
contains a point mutation in the LBD that broadens ligand specificity of the receptor, allowing it to also be activated by progesterone, estradiol, and anti-androgens [89].

Inoculation of LNCaP cells subcutaneously or intraprostatically in male SCID or athymic nude mice results in the formation of a tumour; serum levels of PSA secreted by these tumours correlates with the overall tumour volume [102,103]. Removal of androgens through surgical castration reduces serum levels of PSA, as in the clinical situation. Interestingly, 2 - 4 weeks post-castration the serum PSA levels of these mice exceeds the pre-castration level, indicating that PSA is expressed in an androgen-independent manner in these mice [102,103]. Unlike the clinical situation, though, LNCaP cells are not androgen dependent and thus do not regress after castration [102]. Regardless, LNCaP cells are useful for studying AR-dependent gene expression, both in the presence and the absence of androgens.

The LNCaP xenograft model is thus a valuable system for studying changes in gene expression with the hormonal progression of prostate cancer. However, a disadvantage of this model is that these tumours are highly vascularized and contain a large proportion of murine stromal tissues. Given the high degree of sequence similarity between humans and mice it is difficult to monitor gene expression changes while being confident that only human transcripts are being measured. To address this issue, Sadar et al. developed a novel in vivo prostate cancer tumour model that progresses to androgen-independence upon castration of the host [104]. This model utilizes LNCaP cells grown inside of hollow fibres that are implanted subcutaneously into male nude mice. The fibres that these cells are grown in are porous enough to allow nutrients and wastes to be exchanged, but not so porous as to allow LNCaP cells to escape or mouse cells to enter.
This provides a source of uncontaminated DNA, RNA, and protein extracts for experiments investigating the molecular changes involved in the progression of prostate cancer to androgen-independence.

In addition to LNCaP there are numerous other human prostate cancer cell lines and xenograft models. The two most common in vitro models, besides LNCaP, are the PC3 and DU145 cell lines, both of which are androgen-independent. The PC3 line was derived from a bone metastasis of a human prostate cancer and is more poorly differentiated than LNCaP [105]. These cells do not express AR and are unresponsive to androgens. They also show greater tumourigenicity and a higher growth rate than LNCaP when implanted in male nude mice. The DU145 line was derived from a brain metastasis of a human prostate cancer [106]. These cells are more similar to PC3 in that they also lack a functional AR, are unresponsive to androgens, and show a more malignant phenotype than LNCaP cells when grown as a xenograft. Since neither of these lines express the AR they are not ideal systems in which to study AR-dependent gene expression, nor do they represent the majority of clinical cases of the disease.

An additional model of prostate cancer that has received attention of late is the CWR22 xenograft model. This serially transplantable xenograft was originally derived from a primary prostate cancer [107]. Like LNCaP xenografts, CWR22 xenografts show decreased expression of serum PSA after castration with eventual progression to androgen-independent growth and PSA expression, but on a much slower time scale [108]. Importantly, though, the CWR22 model also regresses after castration, in contrast to the LNCaP model and similar to the clinical situation [109]. However, the androgen-dependent cells are difficult to maintain in vitro, thus limiting the experimental
manipulations which may be performed. And, like the LNCaP subcutaneous xenograft, CWR22 xenografts also contain a significant amount of murine tissues.

While each of the available models of prostate cancer has different advantages, we chose to perform our experiments with the LNCaP hollow fibre xenograft. This decision was made based on availability of the models at the time, our desired experimental designs and need of samples free of mouse tissues, and to give us the flexibility to perform follow-up experiments in either the LNCaP subcutaneous xenograft or LNCaP in vitro models.

1.5 Differential gene expression

A number of genes important in the development of cancer have been identified by first demonstrating altered expression between the normal and cancerous state. Perhaps most famously, the p53 protein was first identified as being upregulated in cells transformed with SV40 T antigen [110]. Of course, p53 is now known to play critical roles in regulating apoptosis, proliferation, and senescence. It has thus become desirable to perform high-throughput gene expression analyses to quickly assay the expression status of large numbers of genes in a given model or treatment condition.

1.5.1 Gene expression changes in prostate cancer

Previous studies have specifically examined gene expression changes in clinical samples of prostate cancer to identify potential markers whose expression levels correlate with patient outcome. For example, patients with decreased expression of KAI1 or E-cadherin were more likely to develop metastatic disease and have poorer overall survival
Likewise, samples of prostate cancer were much more likely to display decreased expression of the glutathione S-transferase gene GSTP1 than were samples of normal tissues [113]. Expression of the cell cycle inhibitor p27 was also shown to be lower in hyperplastic and cancerous prostate tissues relative to normal cells [114]. Samples of early stage prostate cancer were also shown to express increased levels of the kinase PIM1 and the serine protease hepsin [115]. Additionally, tumours expressing increased levels of the MUC1 proteoglycan were more likely to display higher levels of intratumoural angiogenesis [116]. Finally, expression of both EZH2 and BCL2 was shown to be higher in samples of hormone-refractory disease, indicating expression of these proteins may be a marker of hormonal progression [95,117].

However, while increased expression of one of these genes may correlate with one stage of the disease, other stages of the disease may be associated with decreased expression of the same gene. Such results can be difficult to interpret, and consequently, none of these genes have been successfully translated into clinical use. Given this diversity of results, it is interesting to note again the study of Chen et al. [68]. This study examined gene expression changes associated with hormonal progression of prostate cancer in multiple model systems. The final interpretation of their results suggested that increased expression of the AR was the only change consistently shared amongst these models. Importantly, though, these are strictly disease models, whereas the expression changes noted above were observed directly in clinical specimens.

Since focusing on individual genes may be inefficient, other groups have attempted to build prognostic models based on the expression levels of multiple genes in a single sample [118]. Unfortunately, these studies have so far not resulted in altered
clinical management of the disease. Thus, new markers and expression signatures correlating with patient outcome still need to be identified. Interestingly, the identification of novel genes may provide new candidate genes that have not been considered previously.

1.5.2 Techniques for transcriptional profiling

A variety of techniques are now available for monitoring gene expression profiles (see [119] for a review). Microarrays are perhaps the most common method of assaying gene expression of a large number of transcripts. There are two types of microarrays: those spotted with full-length cDNAs of specific genes [120] and those generated with oligonucleotide spots representing regions of known transcripts [121]. Each of these platforms is able to measure the expression of tens of thousands of transcripts. However, microarray experiments are only able to monitor the expression of genes for which prior knowledge of the transcript sequence is available. Microarrays also lack the sensitivity to detect transcripts expressed at very low levels and only provide a relative, not absolute, expression level.

As sequencing capacity increased with the advent of the human genome project some groups began to sequence expressed transcripts as a method of identifying genes. These single-pass sequencing reads, called expressed sequence tags (EST), provided information on what genes were expressed in a sample and was able to identify novel genes [122,123]. However, this method is inefficient both for detecting rare transcripts and for monitoring gene expression changes.
Serial Analysis of Gene Expression (SAGE) is an alternative method that was developed to provide an unbiased and quantitative overview of the entire transcriptome of a sample [124]. It is based on a similar idea as EST sequencing, but SAGE provides for more cost-effective and extensive analyses. SAGE overcomes some of the difficulties of microarrays as this technique does not require prior knowledge of transcript sequence and is thus able to identify and measure the expression level of novel transcripts [124,125]. Additionally, SAGE provides a quantitative measure of gene expression [124]. However, SAGE is an extremely expensive technique requiring significant sequencing capacity. And while SAGE allows for the detection of novel transcripts, the small amount of sequence information in a SAGE tag increases the difficulty of directly studying these novel transcripts.

Differential display was developed in order to specifically amplify and isolate transcripts differentially expressed between two samples [126]. This method also does not require prior sequence knowledge and allows for the isolation of novel differentially expressed transcripts. However, differential display usually identifies only a few candidates, and given the overall complexity of a cDNA sample it would be difficult to isolate low abundance transcripts.

Suppression subtractive hybridization (SSH) is similar to the approach of EST sequencing, but SSH includes a normalization step that enriches for rare transcripts in a population of RNAs, while at the same time selecting against highly abundant transcripts [127,128]. Additionally, subtractive hybridization is able to detect entirely novel transcripts for which no previous annotation exists [129,130]. Thus, subtractive hybridization is a powerful tool to detect less abundant transcripts and the novel
transcripts that tend to be expressed at low levels. In support of this concept, a significant proportion of the transcripts identified by subtractive hybridization were shown to be expressed at levels below the detection limit of Affymetrix GeneChip® microarrays [131]. Subtractive hybridization also identified a number of novel transcripts which were not even represented on these arrays [131], confirming the value of not being dependent on prior knowledge of transcript sequence. However, subtractive hybridization is not as efficient as microarrays or SAGE at generating expression profiles for large numbers of genes. But, in contrast to SAGE, SSH recovers longer sequence fragments, thus simplifying the process of identifying and characterizing novel differentially expressed transcripts.

The wealth of data from the human genome project has greatly changed the means by which high-throughput gene expression analyses are performed. More recent modifications of SAGE and microarrays have also addressed some of their original disadvantages by providing longer sequence tags and higher density arrays to represent a larger proportion of the transcriptome [125]. However, at the time this project began, SSH was the most efficient and cost-effective technique for identifying the novel and low abundance transcripts we were interested in examining.

1.6 Suppression subtractive hybridization

Suppression subtractive hybridization was first described in 1996 by Diatchenko, et al. [127]. The relative advantages and disadvantages of this technique were described in Chapter 1.5. The original subtractive hybridization technique was developed to identify genes expressed under one treatment condition that were not expressed under a
second treatment condition [132]. SSH differs from subtractive hybridization by an additional normalization mechanism that equalizes the relative abundance of cDNAs within a sample, enabling more efficient isolation of transcripts expressed at low levels [127]. The SSH experiments described in this thesis were performed with the PCR-Select™ cDNA Subtraction Kit from Clontech Laboratories, Inc. (Palo Alto, CA). The manufacturer’s protocol and the theory behind the technique are briefly described below.

SSH allows the relative comparison of gene expression levels in two samples of interest. The idea is that one cDNA population (the driver) is “subtracted” from the other cDNA sample (the tester), leaving just those transcripts that are upregulated in the tester sample relative to the driver sample. Reversing this procedure identifies transcripts downregulated in the tester relative to the driver.

First, double-stranded cDNA is generated from the total RNA populations of interest. These cDNAs are then digested with Rsal restriction endonuclease to produce cDNA fragments of approximately equal sizes, thus resulting in similar hybridization efficiencies for all cDNA fragments. Next, the tester cDNA population is split in two groups and different adaptors are ligated to each group (see Fig. 1.3). An excess of driver cDNA is then added to each reaction, the DNA denatured, and hybridization performed. Any cDNA fragments in common between the tester and driver samples will hybridize with each other. High abundance transcripts are more likely to re-anneal with their counterpart cDNAs, thus “normalizing” the concentrations of high and low abundance transcripts.

The two different reactions are then combined and fresh denatured driver is added. Again, the driver cDNA will hybridize with any cDNA fragments also found in
Figure 1.3 – Schematic diagram of suppression subtractive hybridization protocol. Two rounds of subtractive hybridization normalize the relative amount of each cDNA fragment while subtracting cDNA fragments shared between the tester and driver populations. Of the resulting cDNA molecules, only those with unique adaptors at each end are exponentially amplified by PCR. See text for details. Adapted from [127].
the tester sample. As the tester cDNAs are not denatured again, only single-stranded fragments with Adaptor 1 will be free to anneal with their counterpart fragments with Adaptor 2. Thus, these newly annealed molecules have unique adaptors at opposite ends of the double-stranded cDNA. These are also the cDNA molecules that have increased expression in the tester sample relative to the driver sample. Unique PCR primers can then be used to selectively amplify only those cDNA fragments containing different adaptors on either end (see Fig. 1.3). After two rounds of PCR, the differentially expressed genes have been selectively amplified and they can then be cloned and characterized further.

1.7 Summary and research objectives

Prostate cancer is the most frequently diagnosed cancer as well as the second leading cause of cancer deaths among American men. Androgen ablation therapy for patients with advanced prostate cancer inevitably fails as the disease progresses to an androgen-independent stage, leaving patients with no further effective treatment options. It is not understood how prostate cancer cells, normally dependent on androgens for survival, are able to adapt and thrive in this androgen-depleted environment. Current research, though, indicates the androgen receptor is transcriptionally active in many cases of hormone-refractory disease. However, the mechanism(s) by which the AR is activated in the absence of androgen are not fully understood. Additionally, new markers of disease progression are needed to differentiate those patients who will benefit from aggressive treatment from those who will not.
1.7.1 Hypothesis

My overarching hypothesis was that the application of subtractive hybridization at different stages of hormonal progression in the LNCaP hollow fibre model would identify genes of interest in prostate cancer progression and allow for the development of new diagnostic, prognostic, and/or therapeutic targets for the treatment of HRPC. The subtractive hybridization experiments resulted in the isolation of a number of known and novel transcripts associated with the progression of prostate cancer. From these results it was then hypothesized that (1) the characterization of these novel transcripts would identify novel proteins with clinical relevance in the treatment of prostate cancer, and (2) that the known genes isolated here may function in the development of hormone refractory disease.

1.7.2 Specific aims

My specific aims were:

(1) Perform subtractive hybridization to identify the genes expressed at distinct stages of hormonal progression. This work will identify transcripts that may be associated with the development of androgen-independent prostate cancer.

(2) To isolate and identify novel genes expressed during hormonal progression. A significant effort has been expended on examining known genes associated with prostate cancer. This aim will attempt to identify previously uncharacterized genes that are potential markers or therapeutic targets in prostate cancer.

(3) To characterize, where possible, the potential mechanisms by which the identified genes contribute to the hormonal progression of prostate cancer. Some of the
genes identified in Aim 1 may be potential markers of disease progression. However, we also wanted to examine genes that may be responsible for activation of the AR in the absence of androgens.

1.7.3 Thesis overview

The remainder of this thesis describes in detail the experiments I performed to address these specific aims. In Chapter 2, I describe my work with SSH to characterize changes in gene expression during hormonal progression. I examined a novel in vivo tumour model, the LNCaP hollow fibre model, that mimics the hormonal progression of the prostate cancer. This model enabled the isolation and molecular analysis of prostate cancer cells (free from contamination by host cells) at various stages of hormonal progression. Aside from identifying hundreds of known genes, these experiments identified 25 expressed sequences representing novel human transcripts that had not been previously identified. Reverse transcriptase PCR (RT-PCR) for six randomly chosen sequences demonstrated they were expressed in a variety of normal human tissues as well as clinical samples of prostate cancer. Further analysis of two of these novel sequences indicated they were derived from large untranslated regions not previously identified. This study underlined the value of using complementary techniques in the annotation of the human genome.

The work in Chapter 3 was performed to characterize another of the novel expressed sequences described in Chapter 2. This sequence was found to derive from a novel splice variant of the transmembrane protein TMEFF2, which has recently been proposed as a potential immunotherapeutic target for the treatment of prostate cancer.
This variant transcript, expressed by both normal and cancerous prostate cells, was shown to encode a secreted form of the TMEFF2 protein. The potential functions of this isoform and its potential impact on current immune-based treatment strategies are discussed.

As described above, the AR is a ligand-dependent transcription factor that regulates numerous target genes, including PSA. My SSH experiments, as well as experiments performed by other members of the Sadar laboratory, indicated members of the 14-3-3 family of proteins may be differentially expressed with hormonal progression. In Chapter 4, I describe my experiments to examine the ability of each member of this family to modulate transcription of PSA through the AR. Despite significant homology within the 14-3-3 family I observed differences in the ability of each isoform to alter the transcriptional activity of the AR. Significantly, 14-3-3 sigma activated PSA reporters not only at castrate levels of androgens, but also in the complete absence of androgens. Knockdown of the AR by siRNA oligonucleotides abolished activation of these reporters by 14-3-3 sigma, suggesting the AR is required for this activation to occur. I also discuss the potential mechanisms for these findings as well as their potential clinical significance.

Finally, Chapter 5 summarizes all these experiments and discusses the relevance of these findings to prostate cancer research. This chapter also discusses current work and proposes future experiments to build on the results described in this thesis.
1.8 References


66. Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, French FS. Androgen receptor expression in androgen-independent prostate cancer is


111. Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, Debruyne FM, Schalken JA. Decreased E-cadherin expression is associated with


polycomb group protein EZH2 is involved in progression of prostate cancer.


2 NOVEL EXPRESSED SEQUENCES IDENTIFIED IN A MODEL OF ANDROGEN INDEPENDENT PROSTATE CANCER*

2.1 Introduction

Prostate cancer is the most frequently diagnosed cancer as well as the second leading cause of cancer death among American men [1]. Androgen ablation therapy for patients with advanced prostate cancer inevitably fails as the disease progresses to an androgen-independent stage [2]. Few effective treatment options are available to these patients, and these increase survival by only a matter of months [3,4]. We examined an in vivo human prostate cancer tumour model to identify the underlying molecular events involved in hormonal progression. The LNCaP hollow fibre model differs from xenograft models by growing the LNCaP human prostate cancer cell line within fibres that are implanted subcutaneously in host mice [5]. These fibres prevent host cells from infiltrating, and contaminating, the tumour cell population. Upon castration of the host the LNCaP cells progress to an androgen-independent stage as determined by a rising titre of serum prostate-specific antigen (PSA), mimicking this aspect of clinical disease [5].

Many genes important in the development and progression of cancer have been identified by first detecting their altered expression at different stages of the disease. It has thus become desirable to perform high-throughput gene expression analyses to quickly assay the expression status of large numbers of genes in a given model or treatment condition. A variety of techniques are available for monitoring gene

* A version of this chapter is a manuscript in preparation. Quayle, SN, Hare, H, Delaney, AD, Hwang, D, Schein, J, Jones, SJM, Marra, MA, Sadar MD. Novel expressed sequences identified in a model of androgen independent prostate cancer. In preparation.
expression profiles, with microarrays and Serial Analysis of Gene Expression (SAGE) being the most widely used. However, microarray experiments are only able to monitor the expression of genes for which prior knowledge of the transcript sequence is available. Microarrays also lack the sensitivity to detect transcripts expressed at very low levels. The SAGE technique is capable of detecting novel transcripts [6,7], but SAGE is also not optimal for detecting low abundance transcripts. In contrast, suppression subtractive hybridization includes a normalization step that enriches for rare transcripts in a population of RNAs [8,9]. Subtractive hybridization is also able to detect entirely novel transcripts for which no previous annotation exists [10,11]. Thus, subtractive hybridization is a powerful tool to detect less abundant transcripts and the novel transcripts that tend to be expressed at low levels. In support of this concept, a significant proportion of the transcripts identified by subtractive hybridization were shown to be expressed at levels below the detection limit of Affymetrix GeneChip® arrays [12]. Additionally, subtractive hybridization identified a number of novel transcripts which were not represented on these arrays [12].

Gene expression changes occurring with the hormonal progression of prostate cancer have been examined in various systems (see [13-15], for example). Our goal was to utilize the LNCaP hollow fibre model to identify genes that had not been previously associated with prostate cancer in order to gain new insights into the hormonal progression of this disease. The application of subtractive hybridization resulted in the identification of a number of novel expressed sequences in this model of androgen independence. These sequences exhibit low protein-coding potential and low
conservation across species, but RT-PCR experiments confirmed their expression in samples of prostate cancer and in a variety of human tissues.

2.2 Materials and methods

2.2.1 Cell culture

LNCaP cells obtained from Dr. L.W.K. Chung (Emory University School of Medicine, Atlanta, GA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/mL penicillin, and 100 µg/µL streptomycin. All chemicals were purchased from Sigma, unless stated otherwise. Androgen stimulation was performed by treatment with the synthetic androgen R1881 (PerkinElmer, Woodbridge, Canada).

2.2.2 LNCaP Hollow Fibre Model

The LNCaP hollow fibre model has been described in detail previously [5]. Briefly, LNCaP cells (2x10⁷) in RPMI 1640 with 20% (v/v) FBS were sealed inside polyvinylidene fluoride (PVDF) fibres (500 kDa molecular weight cutoff; 1 mm internal diameter; Spectrum Medical Co, Houston, TX) and incubated overnight at 37°C. The fibres were then cut into fragments of approximately 2 cm and inserted subcutaneously into anesthetized male 6-8 week old athymic nude mice (BALB/c strain) obtained from Charles River Laboratory (Montreal, Canada). Serum samples were obtained from the dorsal tail vein of mice every 7 days, and PSA levels measured by an immunoenzymatic assay (Abbott IMX, Montreal, Canada). Serum samples were always obtained prior to the performance of any procedure. After one week mice were castrated by ligation of the
vas deferens through a small incision in the scrotum. Control (intact) animals were not castrated, but all other procedures were performed on the same schedule. Hollow fibres were removed on the day of castration, 10 days post-castration, and 45-60 days post-castration when serum PSA levels had risen. All fibres were immediately placed on ice, washed three times in sterile phosphate buffered saline (PBS), and wiped with sterile, moistened lab wipes. Any fibre that appeared to be contaminated by mouse tissue was set aside. To harvest cells, 1mL of ice-cold TRIZOL® Reagent (Invitrogen, Burlington, Canada) was flushed through the fibres and the cells homogenized with a 21-G needle prior to storage at -80°C. All animal procedures were performed according to protocols approved by the Animal Care Committee at the University of British Columbia (see Appendix).

Two unique sets of hollow fibre samples were analyzed in these experiments. In the first set of samples, the total amount of time the cells were maintained in subcutaneous fibres was kept constant, with all samples being harvested from mice approximately 60 days after implantation of the fibres. Samples were obtained from (A) two mice that were not castrated (intact); (B) one mouse castrated 10 days prior; (C) one mouse castrated 60 days prior that had progressed to androgen independence; and (D) one mouse castrated 61 days prior that had not progressed to androgen independence. Samples B, C, and D were then compared to the intact samples (A) using subtractive hybridization. These samples were all obtained from fibres maintained in different animals.

The second set of samples controlled for any differences that may be observed due to maintenance of fibres in different animal hosts. In this experiment, fibres were
implanted in a single mouse, the mouse was castrated 21 days later, and serum PSA levels in this mouse were deemed to be androgen-independent 63 days after implantation of the fibres. Fibres were removed from this mouse immediately prior to castration (E; 21 days post-implant), 10 days after castration (F; 31 days post-implant), and 42 days after castration (G; 63 days post-implant). Samples E, F, and G were then compared using subtractive hybridization. These samples were all isolated from the same animal, but the cells had been cultivated subcutaneously in hollow fibres for different amounts of time.

### 2.2.3 Suppression subtractive hybridization

The SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) was used to generate full-length cDNA from 1 μg of starting total RNA according to the manufacturer’s protocol. Suppression subtractive hybridization was then performed with the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer’s protocol. Briefly, the cDNA was digested with Rsal restriction endonuclease to generate fragments of approximately equal lengths. The digested cDNA was purified and split into two populations before ligation of Adaptor 1 or Adaptor 2R. An excess of driver cDNA was added to each reaction, the DNA denatured, and hybridization performed for 8 hours at 68°C. The two populations of cDNA were then combined and fresh denatured driver cDNA added before hybridizing for an additional 16 hours. The final cDNA population was subjected to two rounds of PCR to specifically amplify the differentially expressed cDNA transcripts. The efficiency of subtraction was determined by monitoring levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.
(GAPDH) in these samples. The subtracted cDNA pools were then ligated into pCR®2.1-TOPO vector (Invitrogen) and transformed into competent bacteria. Positive colonies were selected and their inserts sequenced unidirectionally with T7 primer on an ABI 3700 automated sequencer. Clones containing novel inserts were sequenced bidirectionally to obtain the entire insert sequence. All sequences obtained in these experiments have been deposited in dbEST (Accession numbers EC093848 - EC094057) and GenBank (Accession numbers DQ668378 - DQ668403).

2.2.4 RT-PCR

Total RNA was isolated from LNCaP cells maintained in vivo using TRIZOL® Reagent according to the manufacturer’s protocol. Total RNA samples from several human tissues were purchased from Stratagene (La Jolla, CA). Samples of total RNA from cases of prostate cancer, and their matched normal samples, were purchased from Genomics Collaborative (Cambridge, MA). Reverse transcription (RT) was performed using MMLV-RT (Invitrogen) with 1 μg of template RNA. Subsequent PCR reactions were performed using 1 μL of the resulting cDNA as template. The primers used to amplify the clones are summarized in Table 2.1. PCR products of interest were cloned into pCR®2.1-TOPO vector and sequenced by the NAPS facility at the University of British Columbia.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B09</td>
<td>F CACAGGAGACCCCTGTCTTACCT</td>
</tr>
<tr>
<td></td>
<td>R AAGCTCTTGCTAGGCATGTAGG</td>
</tr>
<tr>
<td>1E05</td>
<td>F AATAGATGGCAGGCCCTTTG</td>
</tr>
<tr>
<td></td>
<td>R TGGGATGAGCAGGATATCAA</td>
</tr>
<tr>
<td>2A03</td>
<td>F AGAGATGCAAACGGACGAAC</td>
</tr>
<tr>
<td></td>
<td>R TCACCTTACTGCTCTGCAC</td>
</tr>
<tr>
<td>1AC02</td>
<td>F AAGGTTTCCATTGCATCAGG</td>
</tr>
<tr>
<td></td>
<td>R CCTGAAAGGCTGGTCTTCAA</td>
</tr>
<tr>
<td>2DB01</td>
<td>F CCACAACCTTGAAGCAATCA</td>
</tr>
<tr>
<td></td>
<td>R TTCCCTGTCCCTAACTCCT</td>
</tr>
<tr>
<td>2DD06</td>
<td>F TGGCCATCATCAAGTGCA</td>
</tr>
<tr>
<td></td>
<td>R GAGGGATGGTGAATCAGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F CCGAGGCCACATCGCTCAGA</td>
</tr>
<tr>
<td></td>
<td>R CCCAGCCTCTCCATGGTG</td>
</tr>
</tbody>
</table>
2.3 Results

2.3.1 Subtractive hybridization

Suppression subtractive hybridization was used to isolate novel transcripts expressed at different stages of hormonal progression in the LNCaP hollow fibre model. This model enables the isolation and molecular analysis of prostate cells (free from contamination by host cells) at multiple stages of hormonal progression. Samples from intact mice, from mice 10 days after castration (at PSA nadir), and mice 45-60 days post-castration were used as the tester and driver samples in independent subtractive hybridization experiments. Each experiment compared different time points, and both forward and reverse subtractions were performed for each comparison.

Of the known genes identified in these independent experiments, neural cell adhesion molecule 2 (NCAM2) and a disintegrase and metalloproteinase domain 2 (ADAM2) were determined to be upregulated in androgen independence relative to the intact state in both subtractive hybridization experiments. Additionally, peroxiredoxin 3 was found to be increased at 10 days post-castration in both experiments. The identification of these genes in independent experiments supports the conclusion that subtractive hybridization has detected consistent gene expression changes in these samples. Furthermore, the expression of Nkx 3.1, a gene known to be downregulated with prostate cancer progression, was decreased in androgen independence.

2.3.2 Novel transcripts were represented in the subtracted cDNA libraries

To identify novel genes associated with prostate cancer we examined the sequences of all 428 of the subtracted clones isolated from our subtractive hybridization experiments.
These sequences were filtered to remove poor quality sequences and any clones containing less than 25 bp of sequence, more than one insert, or regions of repetitive sequence (Table 2.2). Of the remaining 340 clones, 103 clones contained inserts that were represented by other clones in the library. BLAST analysis of the Ensembl database (v. 35 – Nov 2005) [16] was then used to identify the transcript represented in each of the remaining 237 nonredundant clones. First, the clones were searched against the population of annotated Ensembl cDNAs, identifying 150 clones (63.3%) derived from previously annotated human cDNAs (Fig. 2.1). The remaining 87 clones were then mapped to the human genome using the Ensembl database, with all mappings verified using the UCSC Human Genome Browser (hg17 - May 2004) [17] and GenBank databases. This search identified 57 clones (24.1%) matching expressed sequence tags (EST) and mRNAs which had not yet been classified as Ensembl cDNAs. A clone exhibiting any overlap with a transcript was considered to be part of that transcript. Of the 30 remaining clones, 5 (2.1%) did not align uniquely to the genome, but the other 25 (10.5%) did map uniquely to unannotated regions of the human genome and were considered to represent novel transcripts (Fig. 2.1). These 25 clones mapped to a variety of locations, including within introns of annotated transcripts, nearby annotated transcripts, as well as intergenic regions. This analysis suggested that novel transcripts were represented in our subtractive hybridization libraries.

2.3.3 Characterization of novel clone sequences

We next assessed the sequence characteristics of these novel clones to determine if there were differences between those mapping to unannotated regions of the genome.
Table 2.2 - Summary of sequenced clones.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1 (%)</th>
<th>Experiment 2 (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sequenced clones</td>
<td>211</td>
<td>217</td>
<td>428</td>
</tr>
<tr>
<td>Mouse</td>
<td>23 (10.9)</td>
<td>1 (0.5)</td>
<td>24 (5.6)</td>
</tr>
<tr>
<td>Multiple inserts</td>
<td>2 (0.1)</td>
<td>8 (3.7)</td>
<td>10 (2.3)</td>
</tr>
<tr>
<td>Poor quality</td>
<td>30 (14.2)</td>
<td>24 (11.1)</td>
<td>54 (12.6)</td>
</tr>
<tr>
<td>Redundant</td>
<td>60 (28.4)</td>
<td>43 (19.8)</td>
<td>103 (24.1)</td>
</tr>
<tr>
<td>Remaining Nonredundant</td>
<td>96 (45.5)</td>
<td>141 (65.0)</td>
<td>237 (55.4)</td>
</tr>
<tr>
<td>No unique match</td>
<td>1 (1.0)</td>
<td>4 (2.8)</td>
<td>5 (2.1)</td>
</tr>
<tr>
<td>Annotated gene</td>
<td>74 (77.1)</td>
<td>76 (53.9)</td>
<td>150 (63.3)</td>
</tr>
<tr>
<td>EST</td>
<td>12 (12.5)</td>
<td>45 (31.9)</td>
<td>57 (24.1)</td>
</tr>
<tr>
<td>Unannotated</td>
<td>9 (9.4)</td>
<td>16 (11.3)</td>
<td>25 (10.5)</td>
</tr>
</tbody>
</table>
Figure 2.1 – Distribution of sequence matches in the suppression subtractive hybridization clone set. The 237 non-redundant clones identified through sequence analysis of the subtractive hybridization libraries were mapped to the human genome (v. 35) and classified as representing an annotated Ensembl cDNA, a previously identified expressed sequence tag (EST), or an unannotated region of the genome. Clones not mapping uniquely to the genome were classified as ambiguous matches.
relative to those clones mapping to known transcripts. Five characteristics were examined for each clone: (1) their protein coding potential, (2) their evolutionary conservation, (3) whether they showed evidence of splicing, (4) whether they demonstrated homology to known non-coding RNAs, and (5) whether they contained a poly-adenylation site. For this analysis, the 25 novel clones (Table 2.3) were compared to 25 clones randomly chosen from the 57 that mapped to previously identified ESTs (Table 2.4), and another 25 clones randomly chosen from the 150 mapping to annotated transcripts (Table 2.5). First, to assess the likely coding potential of each clone, the length of the longest open reading frame (ORF) was determined using the ORF Finder tool at NCBI. The clones derived from known transcripts were much more likely to contain an ORF spanning most of the sequence. It should also be noted that the sequences used for this analysis were derived from single-pass sequencing reads, and thus potential sequencing errors may interrupt longer ORFs. Next, we qualitatively assessed the degree of conservation of each of these clones using the Vertebrate and Multiz Alignment and Conservation track at UCSC [18]. This track provides a measure of evolutionary conservation of a genomic region amongst 17 vertebrate species. As predicted, clones originating from known transcripts tended to show high conservation of their respective genomic loci, while the clones representing novel transcripts mapped to regions of the genome that were poorly conserved. This was consistent with the prediction that highly conserved transcripts would be expressed by a larger number of species, thus increasing the likelihood of the transcript having been detected in previous studies.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession</th>
<th>Length (bp)</th>
<th>Open Reading Frame (%)</th>
<th>Degree of Conservation</th>
<th>Splicing</th>
<th>polyA++</th>
</tr>
</thead>
<tbody>
<tr>
<td>11_B09</td>
<td>DQ668378</td>
<td>306</td>
<td>25.2</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>11_E05</td>
<td>DQ668379</td>
<td>1338</td>
<td>8.1</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12_A03</td>
<td>DQ668380</td>
<td>543</td>
<td>46.8</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12_A06</td>
<td>DQ668381</td>
<td>324</td>
<td>39.2</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12_E10</td>
<td>DQ668383</td>
<td>576</td>
<td>46.7</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>13_F09</td>
<td>DQ668384</td>
<td>454</td>
<td>50.7</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>13_G01</td>
<td>DQ668385</td>
<td>261</td>
<td>41.0</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>13_G06</td>
<td>DQ668386</td>
<td>389</td>
<td>32.1</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>14_D01</td>
<td>DQ668387</td>
<td>134</td>
<td>77.6</td>
<td>+++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_A04</td>
<td>EC093934</td>
<td>245</td>
<td>25.3</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_C02</td>
<td>DQ668388</td>
<td>1853</td>
<td>12.4</td>
<td>++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_E09</td>
<td>DQ668389</td>
<td>796</td>
<td>29.5</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1b_C12</td>
<td>DQ668390</td>
<td>555</td>
<td>16.8</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1c_D03</td>
<td>DQ668391</td>
<td>292</td>
<td>18.5</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1c_E10</td>
<td>DQ668392</td>
<td>679</td>
<td>14.9</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1c_F02</td>
<td>DQ668393</td>
<td>475</td>
<td>26.7</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_E01</td>
<td>DQ668394</td>
<td>771</td>
<td>26.7</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_F04</td>
<td>DQ668395</td>
<td>465</td>
<td>21.9</td>
<td>+++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2c_A06</td>
<td>EC094025</td>
<td>658</td>
<td>10.8</td>
<td>++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2c_C10</td>
<td>DQ668396</td>
<td>371</td>
<td>28.0</td>
<td>++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2c_G10</td>
<td>DQ668397</td>
<td>969</td>
<td>19.8</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_B01</td>
<td>DQ668398</td>
<td>1100</td>
<td>15.2</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_D06</td>
<td>DQ668399</td>
<td>598</td>
<td>37.0</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2d_D09</td>
<td>EC094055</td>
<td>627</td>
<td>26.6</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_E01</td>
<td>DQ668400</td>
<td>722</td>
<td>34.8</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

† Length of longest open reading frame as a percentage of the total length of each clone.

* Degree of conservation of each clone, +, little to no conservation; ++++, high degree of conservation.
Table 2.4 - Sequence characteristics of clones matching previously identified ESTs.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession</th>
<th>Length (bp)</th>
<th>Open Reading Frame (%)</th>
<th>Degree of Conservation*</th>
<th>Splicing</th>
<th>polyA++</th>
</tr>
</thead>
<tbody>
<tr>
<td>11_C08</td>
<td>EC093853</td>
<td>470</td>
<td>19.6</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>13_F07</td>
<td>EC093910</td>
<td>281</td>
<td>44.5</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>13_G08</td>
<td>EC093913</td>
<td>126</td>
<td>75.4</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>14_D02</td>
<td>EC093923</td>
<td>671</td>
<td>27.6</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>14_G04</td>
<td>EC093933</td>
<td>521</td>
<td>42.4</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_B02</td>
<td>EC093936</td>
<td>579</td>
<td>25.7</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_D01</td>
<td>EC093941</td>
<td>451</td>
<td>20.4</td>
<td>++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1a_F05</td>
<td>EC093946</td>
<td>478</td>
<td>19.9</td>
<td>+++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1a_H09</td>
<td>EC093956</td>
<td>683</td>
<td>33.7</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1b_A09</td>
<td>EC093959</td>
<td>619</td>
<td>30.4</td>
<td>+++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1b_A11</td>
<td>EC093961</td>
<td>284</td>
<td>63.0</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1b_C01</td>
<td>EC093963</td>
<td>584</td>
<td>27.9</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1c_A02</td>
<td>EC093968</td>
<td>514</td>
<td>25.5</td>
<td>++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1c_D12</td>
<td>EC093979</td>
<td>215</td>
<td>59.5</td>
<td>+++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1c_E05</td>
<td>EC093980</td>
<td>429</td>
<td>49.4</td>
<td>+</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>1c_G06</td>
<td>EC093989</td>
<td>525</td>
<td>26.1</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_C12</td>
<td>EC094007</td>
<td>596</td>
<td>22.0</td>
<td>++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1d_G02</td>
<td>EC094018</td>
<td>351</td>
<td>36.5</td>
<td>+++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2c_B04</td>
<td>EC094028</td>
<td>484</td>
<td>21.5</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2c_B10</td>
<td>EC094032</td>
<td>427</td>
<td>44.0</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2c_D10</td>
<td>EC094037</td>
<td>487</td>
<td>37.0</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2c_F04</td>
<td>EC094043</td>
<td>384</td>
<td>45.8</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2c_H03</td>
<td>EC094044</td>
<td>642</td>
<td>48.0</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_B10</td>
<td>EC094049</td>
<td>299</td>
<td>54.8</td>
<td>+++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_D07</td>
<td>EC094054</td>
<td>299</td>
<td>60.5</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

* Degree of conservation of each clone, +, little to no conservation; +++++, high degree of conservation.

† Length of longest open reading frame as a percentage of the total length of each clone.
Table 2.5 - Sequence characteristics of clones matching previously annotated cDNAs.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession</th>
<th>Length (bp)</th>
<th>Open Reading Frame (%)</th>
<th>Degree of Conservation</th>
<th>Splicing</th>
<th>polyA++</th>
</tr>
</thead>
<tbody>
<tr>
<td>11_F09</td>
<td>EC093861</td>
<td>149</td>
<td>100.0</td>
<td>+</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>11_G07</td>
<td>EC093866</td>
<td>169</td>
<td>86.4</td>
<td>+++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>11_G09</td>
<td>EC093868</td>
<td>323</td>
<td>43.3</td>
<td>++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>12_F01</td>
<td>EC093899</td>
<td>426</td>
<td>91.3</td>
<td>+</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>12_F10</td>
<td>EC093893</td>
<td>276</td>
<td>74.6</td>
<td>+++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>12_G09</td>
<td>EC093898</td>
<td>279</td>
<td>49.1</td>
<td>++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12_G11</td>
<td>EC093899</td>
<td>433</td>
<td>73.2</td>
<td>++++</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>14_C11</td>
<td>EC093921</td>
<td>269</td>
<td>94.1</td>
<td>+++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>14_D07</td>
<td>EC093926</td>
<td>77</td>
<td>96.1</td>
<td>++++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>14_E12</td>
<td>EC093929</td>
<td>318</td>
<td>43.1</td>
<td>+++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>14_F09</td>
<td>EC093932</td>
<td>180</td>
<td>100.0</td>
<td>+++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>1a_B07</td>
<td>EC093937</td>
<td>477</td>
<td>69.0</td>
<td>++++</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>1a_E11</td>
<td>EC093945</td>
<td>655</td>
<td>33.7</td>
<td>++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>1c_B12</td>
<td>EC093972</td>
<td>555</td>
<td>32.3</td>
<td>+++</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>1c_D05</td>
<td>EC093975</td>
<td>325</td>
<td>27.4</td>
<td>+++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1c_D07</td>
<td>EC093977</td>
<td>425</td>
<td>37.9</td>
<td>++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_B08</td>
<td>EC094000</td>
<td>408</td>
<td>34.3</td>
<td>++++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_D04</td>
<td>EC094008</td>
<td>245</td>
<td>100.0</td>
<td>++++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>1d_E07</td>
<td>EC094012</td>
<td>555</td>
<td>89.2</td>
<td>++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>1d_E11</td>
<td>EC094014</td>
<td>670</td>
<td>60.6</td>
<td>++</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1d_F08</td>
<td>EC094016</td>
<td>297</td>
<td>91.9</td>
<td>++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>1d_H01</td>
<td>EC094020</td>
<td>701</td>
<td>44.8</td>
<td>++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2c_B03</td>
<td>EC094027</td>
<td>92</td>
<td>100.0</td>
<td>++++</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2c_D08</td>
<td>EC094036</td>
<td>475</td>
<td>37.7</td>
<td>++++</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2d_B03</td>
<td>EC094046</td>
<td>388</td>
<td>29.9</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

† Length of longest open reading frame as a percentage of the total length of each clone.
* Degree of conservation of each clone, +, little to no conservation; ++++, high degree of conservation.
We were also interested in whether these sequenced clones derived from spliced regions of transcripts. While over half of the annotated clones appeared to be spliced, only one clone matching an EST was spliced, and none of the novel clones showed evidence of splicing. It may be that the clones matching ESTs and unannotated regions were more frequently derived from untranslated regions of transcripts. This would be consistent with the decreased size and number of ORFs in these clones. A number of families of non-coding RNAs have also now been identified, such as ribosomal RNAs, transfer RNAs, and more recently, micro RNAs. To determine if the unannotated sequences identified here may be non-coding RNAs, these sequences were queried against the Rfam database of non-coding RNA families [19]. None of the novel sequences displayed homology to any of the known RNA families in this database. Next we examined all these clones for the presence of poly-adenylation sites. Approximately 30% of all clones, regardless of their mapping, were poly-adenylated, suggesting the novel clones were derived from mRNA transcripts. This result also showed that the known and novel transcript fragments were equally likely to contain poly-adenylation sites, suggesting that novel clones were not specifically biased towards the 3' untranslated regions of transcripts.

2.3.4 Analysis of SAGE tags in the novel clones

SAGE is a second technique that has been used to identify large numbers of novel transcripts [6,7]. We sought to determine if the 25 novel clones had been previously detected in SAGE experiments performed with human tissues. Subtracted clones matching unannotated regions of the human genome were searched for potential NlaIII
restriction sites and the 17 bp of downstream sequence was considered to represent a LongSAGE tag. Twenty of our unannotated sequences contained uniquely mapping regions which could be potential LongSAGE tags, and many clones contained multiple possible tags. We then compared our predicted LongSAGE tags to a metalibrary containing approximately 11 million uniquely mapping human SAGE tags previously identified at the B.C. Cancer Agency Genome Sciences Centre or in the Cancer Genome Anatomy Project (CGAP) at NCBI. These tags were identified in 79 SAGE libraries representing a variety of human tissues, including embryonic and haematopoietic stem cells, samples of normal and cancerous lung, brain, pancreas, breast and colon, as well as some human cancer cell lines. Only three of the 20 unannotated clones contained LongSAGE tags that were previously detected in a SAGE experiment; none of these tags mapped to annotated transcripts. These tags, from clones 1cE10, 2dB01, and 2dD06, occurred once, thrice, and once, respectively, in the entire human LongSAGE metalibrary, providing in silico evidence that these clones represent novel transcripts that are expressed at very low levels.

A LongSAGE tag and a novel clone may also derive from the same transcript without the two sequences directly overlapping. We therefore looked for LongSAGE tags mapping to the 50 kb of genomic DNA sequence flanking our novel sequences to identify any neighbouring regions predicted to be expressed based on the previous SAGE experiments. The genomic neighbourhoods of all 20 of the analyzed subtracted clones contained LongSAGE tags that were not from annotated transcripts. As stated above, 3 of these clones mapped directly to a LongSAGE tag. Of the remaining 17 clones, 7 mapped within 2.5 kb of a previously detected LongSAGE tag, while the remaining 10
clones were up to 23 kb from their nearest tag. These tags also occurred at low frequency in the human metalibrary, with each tag being detected from one to three times. These regions of the genome do not appear to encode abundantly expressed transcripts, providing a possible explanation why these transcripts were not detected previously. This analysis supported the concept that our novel subtracted clones were likely to represent entirely novel transcripts.

2.3.5 The novel subtracted clones are expressed by a variety of cell types

To confirm that the novel clones detected by subtractive hybridization are naturally expressed in cells we performed a series of RT-PCR experiments. We chose 6 novel clones and examined their expression in LNCaP cells from the hollow fibre model. All six of the chosen clones were clearly expressed in LNCaP cells (Fig. 2.2A). The absence of bands in the reverse transcriptase (RT)-negative samples confirmed that these products did not derive from contamination with genomic DNA. The tested clones included two clones containing a previously identified LongSAGE tag, three clones for which the nearest tag was between 2 to 8 kb away, and one clone that was approximately 14 kb from the nearest LongSAGE tag. Thus, the novel transcript fragments isolated here were derived from expressed transcripts that had not previously been documented in any database.

To determine the tissue distribution of these novel transcripts we performed RT-PCR with a panel of RNAs derived from "normal" human tissues. Each of the 6 clones chosen displayed unique tissue expression profiles, with 4 clones being highly specific to the prostate and testes or the testes alone, while the remaining 2 clones were expressed in
Figure 2.2 – The novel subtracted clones were expressed in a variety of human tissues.

(A) RT-PCR was performed with primer pairs specific for each of six novel clones using cDNA from the LNCaP Hollow Fibre Model. RT indicates the presence or absence of Reverse Transcriptase. (B) RT-PCR was performed using cDNAs generated from normal human tissues with each of the six novel clones. All clones were expressed in at least one of the normal tissues. (C) RT-PCR was performed using cDNAs generated from three samples of prostate cancer (T1 - T3) and their respective matched normal samples (N1 - N3). All six of the clones were expressed in at least one of these samples.
all the tissues tested (Fig. 2.2B). Clone 2A03 was almost exclusively expressed in the prostate, with only a faint product observed in the testes. Even the two clones observed in all the tissues tested appeared to be expressed at variable levels in each of the tissues. Thus, while the expression of some of these clones appeared higher in LNCaP cells, the expression of these novel sequences was not unique to this cell line.

Next we examined the expression of these 6 novel transcripts in three samples of prostate cancer with matched samples of “normal” prostate. While the cancer specimens were not microdissected, these samples were all scored by a pathologist to contain 65-80% tumour tissue. Five of the transcripts were expressed in both the normal and cancer specimens (Fig. 2.2C). Clone 2dB01 showed the most limited expression, being detectable in only one sample of cancer. The expression of this transcript is quite limited as LNCaP cells and the single cancer specimen were the only non-testicular samples in which expression could be demonstrated (Fig. 2.2B, C). While expression levels can not be precisely measured via RT-PCR, some of these clones appeared to show differential expression in the normal samples relative to the cancer samples. From these experiments we conclude that the novel clones derived from our subtractive hybridization experiments in fact represent previously unannotated transcripts that are expressed in a variety of human tissues.

2.3.6 Two of the novel clones are part of larger untranslated regions

The novel clones isolated here were cDNA fragments presumably derived from larger transcripts. We wanted to isolate more sequence information from the full-length transcripts in order to understand the possible functions of these novel transcripts. The
above analysis with the human SAGE metalibrary identified a number of tags mapping near the novel clones isolated using subtractive hybridization, suggesting they may represent the same transcript. RT-PCR experiments were then performed utilizing primers spanning clones 1E05 and 2dB01 and their neighbouring LongSAGE tags. These experiments demonstrated that some of these tags were in fact derived from larger transcripts that also contained these novel subtracted clones (Fig. 2.3). These PCR products were not detected in reverse transcriptase negative samples, confirming they were not derived from genomic DNA. Upon sequencing of these PCR amplicons, clone 1E05 was demonstrated to be part of an additional 6 kb untranslated region (accession nos. DQ668402 and DQ668403) downstream of a previously identified, but uncharacterized, mRNA represented in the GenBank database (AL832227). Interestingly, mRNA AL832227 was originally identified in human testis tissue. This provides validation of the tissue expression profiles we observed as clone 1E05 was only detected in cDNA from testis (Fig. 2.2B). Finally, clone 2dB01 was part of a 1.6 kb transcript that did not contain any significant ORFs (accession no. DQ668401). This transcribed region is likely an untranslated region of a larger transcript that has yet to be identified.

2.4 Discussion

While the precise number of genes in the human genome remains unknown, it is clear that an even greater number of transcripts are produced by a myriad of alternative splicing events. The recognition of non-coding RNAs has also resulted in a greater focus on transcripts not containing open reading frames. Thus, even with the completed
Figure 2.3 – Two of the novel clones were part of transcripts containing a neighbouring SAGE tag. RT-PCR amplified transcript regions between clones 1E05 (A) and 2dB01 (B) and SAGE tags identified nearby each clone. RT indicates the presence or absence of Reverse Transcriptase.
sequence of the genome further experiments are required to fully annotate the functional units transcribed from the genome in the different cell types during normal growth and development, as well as in diseased tissues. High-resolution tiling microarrays have been used to generate predictions of likely transcripts, many of which mapped to intergenic and intronic regions of the genome that were not previously annotated [20-22]. Similarly, approaches focused on sequencing full-length cDNAs continue to identify a large number of novel transcripts, many of which appear to be non-protein coding [23,24]. Our results here provide further evidence that a significant number of genes and transcripts remain to be identified.

Studies now suggest that the majority of the human genome is transcribed, but the function(s) of most of these transcripts has not yet been demonstrated (reviewed in [25]). The lack of defined function for these transcripts has led some to propose that they arise indirectly through spurious transcription of the genome. However, new functions of non-coding transcripts continue to be identified, indicating that these "spurious" transcripts likely have functions that just have not been characterized. For example, steroid receptor RNA activator (SRA) was demonstrated to act as an RNA transcript to regulate the transcriptional activity of steroid nuclear receptors, including the androgen receptor [26]. Another non-coding transcript, expressed at low levels in various tissues, was recently demonstrated to regulate the nuclear trafficking of nuclear factor of T cells (NFAT), and has been renamed non-coding RNA repressor of NFAT (NRON) [27]. Such studies confirm that low-abundance, non-coding, RNA transcripts perform diverse functions and regulate multiple biological processes.
Subtractive hybridization has been used to characterize gene expression changes associated with prostate cancer [10,13,28-31], and some of these studies have also characterized novel genes that were identified from their subtracted libraries [10,11,28]. However, these reports examined only a few novel genes that were differentially expressed in their experimental systems, and few studies have used subtractive hybridization to examine changes with hormonal progression in an \textit{in vivo} model [13].

Our study is unique in that we sequenced all the clones arising from the subtractive hybridization experiments performed with \textit{in vivo} samples from the LNCaP hollow fibre model. This approach identified a large number of transcripts that had not previously been detected in prostate cancer cells and may be of prognostic or therapeutic value.

We identified 25 completely unannotated clones, and an additional 57 clones matching previously sequenced ESTs that were otherwise unannotated. The isolation of these ESTs specifically from prostate cancer cells may prove informative at a later date. Furthermore, we considered any overlap of an EST or annotated gene with one of our clones to signify that that clone derived from a previously identified transcript. However, several of the clones matching ESTs and annotated transcripts displayed only partial overlap with the known sequence, suggesting that the subtracted clone may still represent an unidentified splice variant of the known transcript.

The functional relevance of these novel transcripts in the hormonal progression of prostate cancer remains to be elucidated. In our experiments, 4 of the 6 clones tested were expressed only in the normal prostate and testes; this limited tissue expression profile suggests these novel transcripts may function specifically in these organs. This also suggests the expression of these transcripts is tightly regulated, as would be expected.
for a functional transcript. Furthermore, a related publication from our group identified a novel variant of TMEFF2 which encodes a secreted form of the protein [32]. This alternate form of the protein was identified after a novel clone from our subtractive hybridization library (clone 2A06) was mapped to the fourth intron of the TMEFF2 gene. The expression of TMEFF2 has been shown to increase with progression to androgen independence [13,33], consistent with our subtractive hybridization experiments. TMEFF2 is also currently being investigated as a target for antibody-based therapy in the treatment of prostate cancer [34,35], confirming that our approach identified novel transcripts which may be of interest in the study of prostate cancer. Changes in expression of some of these transcripts may also be valuable as a marker for disease progression.

To characterize the function(s) of these novel transcripts it will first be necessary to identify the full-length cDNA sequence. Multiple techniques are available to recover full-length cDNA molecules starting from only cDNA fragments. For example, Rapid Amplification of cDNA Ends (RACE) is widely used to obtain full-length cDNA sequences [36]. However, this technique is most effective when enough sequence information is available to enable the design of nested PCR primers and when the orientation of that sequence is known. We performed preliminary RACE experiments with some of our novel subtracted clones, but the efficiency of these experiments was low as evidenced by the large number of non-specific amplicons that were produced (data not shown). This may be partially due to the inability to determine the genomic orientation of subtractive hybridization clones, necessitating the consideration of all possible transcripts when designing RACE experiments. Another technique to identify
full-length sequence for these novel transcripts would be to screen existing cDNA libraries [6,11]. However, given the relatively low expression of these transcripts this approach would likely require extensive screening of such libraries.

Margulies et al. [37] recently described a highly parallel sequencing by synthesis approach that demonstrated an increased throughput for sequencing of genomic DNA. Our group has combined sequencing by synthesis and random shotgun analysis to generate ESTs and characterize the transcriptome of LNCaP cells grown in tissue culture (MN Bainbridge et al, submitted). This study isolated approximately 180,000 ESTs, and of these, 1,900 (1.0%) mapped to the human genome in regions not previously annotated in the Ensembl database. One of these ESTs mapped directly to clone 2A03 described here, while 12 more mapped within 1.5 kb of clones 1cD03 and 2dB01. However, the remaining 22 novel clones identified here were still not detected by this alternative approach.

SAGE has also been used to identify potentially novel transcripts [6,7,38]. Unfortunately, while SAGE provides sufficient sequence information to accurately map the tags to the human genome, there is often little other information available to aid in the design of experiments to derive more sequence data from these novel transcripts. In contrast, subtractive hybridization provides longer sequence fragments, but it is not possible to determine the orientation of these fragments. Our data also suggest that subtractive hybridization was able to detect transcripts that had not previously been found using SAGE or high throughput sequencing by synthesis. It is possible that subtractive hybridization may be more sensitive to detecting transcripts expressed at low levels. Alternatively, subtractive hybridization may isolate those transcripts that can not be
efficiently detected by SAGE, for instance transcripts lacking an NlaIII restriction site. Common results from multiple techniques gives greater confidence in the identification of novel transcripts and underlines the value of using complementary techniques to achieve a more thorough analysis of the human transcriptome. In conclusion, our subtractive hybridization experiments have identified novel transcripts that are specific for the prostate and/or the urogenital tissues. It may be of clinical value to further develop these novel transcripts as prognostic or therapeutic markers for prostate cancer and hormonal progression.
2.5 References


3 A TRUNCATED ISOFORM OF TMEFF2 ENCODES A SECRETED PROTEIN IN PROSTATE CANCER CELLS

3.1 Introduction

The human TMEFF2 (transmembrane protein with EGF-like and two follistatin-like domains 2) gene was initially described by a number of independent groups [1-6]. Together, these reports described TMEFF2 as a proteoglycan predominately expressed in the brain and prostate. TMEFF2 was also shown to be hypermethylated in bladder and colorectal tumour cells. While its full name illustrates its predicted domains, a physiological role for TMEFF2 has not been determined. Overexpression of full-length TMEFF2 inhibited the proliferation of two aggressive prostate cancer cell lines [6] while the soluble extracellular domain of TMEFF2 increased survival of neuronal cells in primary cultures [3]. Additionally, the TMEFF2 extracellular domain elicited weak tyrosine phosphorylation of erbB-4 [1]. TMEFF2 has received recent attention as a possible immunotherapeutic target for the treatment of metastatic prostate cancer [7,8]. This interest is due to its restricted expression profile as well as its increased expression in androgen-independent prostate cancer [5,9]. We report here the identification of a novel splice variant of TMEFF2 that encodes a secreted protein (TMEFF2-S).

3.2 Materials and methods

3.2.1 Cell culture and transfection

LNCaP cells obtained from Dr. L.W.K. Chung (Emory University School of Medicine, Atlanta, GA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal
bovine serum (FBS) (HyClone, Logan, UT), 100 units/mL penicillin, and 100 µg/µL streptomycin (Invitrogen, Burlington, Canada). All chemicals were purchased from Sigma, unless stated otherwise. Androgen stimulation was performed by treatment with the synthetic androgen R1881 (10 nM, PerkinElmer, Woodbridge, Canada) for 24hr. For transfections, LNCaP cells were plated at 3.0x10^5 cells per well in 6-well plates. After 24 hr, transfections were performed using LIPOFECTIN® Reagent (Invitrogen) according to the manufacturer's protocol. Plasmids encoding full-length or truncated TMEFF2 were transfected at 1 µg and 3 µg/well in separate wells. The total amount of transfected DNA was normalized to 3 µg/well by the addition of pRc-CMV (Invitrogen) plasmid encoding the CMV promoter but lacking a downstream gene. After 24 hr RPMI 1640 was added to the cells to a final concentration of 5% FBS.

3.2.2 RT-PCR

Total RNA was isolated from LNCaP cells maintained in vivo [10] or in vitro, PrEC cells (Clonetics, Guelph, Canada), or PC3 prostate cancer cells using TRIZOL® Reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA samples from several normal human tissues were purchased from Stratagene (La Jolla, CA). Total RNA from clinical specimens of prostate cancer, and their matched normal samples, was purchased from Genomics Collaborative (Cambridge, MA). Reverse transcription (RT) was performed using MMLV-RT (Invitrogen) with 1 µg of template RNA. Subsequent PCR reactions were performed using 1 µL of the resulting cDNA as template. Primers used to amplify TMEFF2 and EST 2A06 were as follows: Primer 1, 5'-'CCCGTCATGCTACTCATCGT-3'; Primer 2, 5'-'
CTGAACAAAAGAGTGAAGGGAAC-3'; Primer 3, 5'-
CCCTTGTGCAAATGGGTTA-3'; and Primer 4, 5'-AATGCTCACACTTCCCATGC-3'. The primers used to detect GAPDH were: Forward 5'-
CCGAGCCACATCGCTCAGA-3' and Reverse 5'-CCCAGCCTTCTCCATGGTG-3'.

3.2.3 Plasmids

A Mammalian Gene Collection expression clone (Clone ID 4180709) containing the entire transcript for TMEFF2 was purchased from Invitrogen. The open-reading frames of full-length and truncated TMEFF2 were cloned into pcDNA3.1™D/V5-His-TOPO® vector (Invitrogen) using the forward primer 5'-
CACCATGGTGCTGTGGGAGTCC-3'; TMEFF2-reverse 5'-
GATTAACCTCGTGGACGCTC-3'; and TMEFF2-S-reverse 5'-
AATATCTGAAATGGAACATAC-3'. The same forward primer was used to amplify both open reading frames. These inserts were then removed by digesting with HindIII and EcoRV restriction endonucleases, followed by ligation into the HindIII and Smal sites of the pEGFP-N3 vector. All plasmids were sequence verified.

3.2.4 Western blot analysis

LNCaP cells were harvested and washed twice in ice-cold PBS. Cell pellets were lysed in 2x SDS-PAGE loading buffer and boiled for five minutes prior to loading on 12% SDS-PAGE gels. Media from transfected cells was removed after incubation for 24 hours, centrifuged to remove unattached cells, and 25 μL samples were analyzed on 12% SDS-PAGE gels. The separated proteins were transferred to nitrocellulose membranes.
(Amersham Biosciences, Piscataway, NJ) and blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for one hour at room temperature or at 4°C overnight, and then washed three times. The blots were probed with anti-GFP (B-2)(1:500)(Santa Cruz Biotechnology) or anti-β-actin (ab6276)(1:5000)(Abcam, Cambridge, MA) for 1 to 2 hours at room temperature, washed three times, and incubated with the appropriate IR-labelled secondary antibody (1:5000, Molecular Probes) for 30 minutes at room temperature. All washes were performed with PBS containing 0.1% Tween20. Primary antibodies were diluted in Odyssey Blocking Buffer with 0.1% Tween20, while secondary antibody dilutions contained an additional 0.01% SDS. Visualization was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences).

3.2.5 Fluorescent microscopy

LNCaP cells (3.0x10^5 cells per well) were plated on sterile coverslips in 6-well plates. Transfections were performed as described above. The cells were fixed for 30 minutes in 4% paraformaldehyde at room temperature, followed by two washes in sterile PBS. Nuclei were counterstained with 300 nM 4',6-Diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature. The cells were again rinsed in sterile PBS prior to mounting on glass slides. Images were captured using an AxioCam high resolution colour camera on an AxioPlan 2 fluorescent microscope (Carl Zeiss, Toronto, Canada). Images of the GFP and DAPI channels were merged using Adobe Photoshop software.
3.3 Results

3.3.1 A novel splice variant is transcribed from the TMEFF2 locus

Previous experiments in our laboratory have generated a collection of novel expressed sequence tags (EST; see Chapter 2) derived from a LNCaP xenograft model of prostate cancer [10]. Sequencing of one such EST (referred to as EST 2A06) revealed a 330bp sequence with 99.1% homology to a region of chromosome 2q32.3. Upon further analysis, EST 2A06 was mapped to the fourth intron of the gene TMEFF2 (also known as tomoregulin-2, HPP1, or TENB2; Fig. 3.1A). RT-PCR with primers anchored in the known transcript and EST 2A06 amplified an unidentified product of approximately 3.3 kb, indicating EST 2A06 represented a novel splice variant of the TMEFF2 gene (Fig. 3.1B). However, the cDNA identified did not include the 3’ region of the known TMEFF2 transcript. Sequencing of the 3.3 kb band revealed a cDNA encoding the first four exons of TMEFF2 followed by 2.9 kb of the fourth intron (GenBank accession number DQ133599). A predicted translation of this cDNA suggested it encoded a 175 amino acid open reading frame (ORF) consisting of the first 146 residues of TMEFF2 followed by 29, mostly hydrophobic, residues not found in the normal TMEFF2 protein sequence. These 29 novel amino acids (GRSCLFTYLKIYWWILLCIFTYVCSISDI) did not exhibit significant similarity to any proteins in GenBank. Significantly, this predicted isoform, designated TMEFF2-S, lacked one follistatin-like domain, the EGF-like domain, and the transmembrane domain (Fig. 3.1C).
Figure 3.1 - A novel EST is part of a larger transcript derived from the TMEFF2 locus.

(A) Schematic cartoon summarizing the TMEFF2 genomic locus (transcribed from left to right) and the relative location of the sequenced EST 2A06 in the fourth intron (not to scale). Horizontal lines represent introns, vertical lines represent exons, and filled boxes represent untranslated regions (UTRs). Numbered arrows represent the locations of the primers used in (B). (B) Results of RT-PCR experiment using the primer pairs from (A) indicated above each lane. These results suggest EST 2A06 and TMEFF2 share a single transcript. (C) Schematic cartoon showing the domain structure of the peptide predicted from the sequence of the novel splice variant. SS, signal sequence; FS, follistatin-like domain; EGF, EGF-like domain; TM, transmembrane domain; N, novel amino acid residues.
3.3.2 TMEFF2-S was expressed in normal human tissues and produced a truncated protein

To determine the tissue distribution of TMEFF2-S, we performed RT-PCR with cDNA samples obtained from the prostatic cell lines LNCaP, PrEC, and PC3, from normal human tissues, and from samples of prostate cancer and their matched normal samples (Fig. 3.2). While the prostate cancer specimens were not microdissected, they did contain 65-80% tumour tissue. Additionally, tumour sample T3 expressed the TMEFF2-S transcript while the matched normal sample N3 showed little to no expression of this transcript, suggesting that in this case TMEFF2-S was only expressed by the cancer cells (Fig. 3.2C). No bands were detected in the reverse-transcriptase (RT) negative samples, indicating this product was not due to contamination with genomic DNA. Identical results were obtained from PCR with two other primer pairs located in different regions of the TMEFF2-S transcript. Expression of this isoform was limited to LNCaP cells, normal prostate, and testis. Importantly, TMEFF2-S expression was also observed in prostate cancer specimens.

To demonstrate that this splice variant produced a truncated protein as predicted from its cDNA sequence, GFP was fused to the carboxyl terminus of both full-length TMEFF2 and TMEFF2-S. Each isoform was transfected into LNCaP cells and expression of the fusion proteins monitored by western blot analysis of whole cell protein lysates using an anti-GFP antibody. As predicted, the full-length TMEFF2-GFP fusion protein was approximately 75 kDa (Fig. 3.3); there were, however, other products of this fusion protein, most likely due to post-translational modifications. The extracellular domain of TMEFF2 was previously shown to be cleaved at multiple sites, leaving small
Figure 3.2 - The novel splice variant of TMEFF2 is expressed in normal tissues. RT-PCR was performed with primers located within EST 2A06 using cDNA from LNCaP, PrEC, and PC3 cell lines (A), from normal human cDNAs (B), and from three samples of prostate cancer (T1, T2, T3) and their respective matched normal samples (N1, N2, N3) (C). RT-PCR for GAPDH confirmed the presence and quality of cDNA in each sample. The novel TMEFF2 transcript was expressed specifically in prostate-derived tissues. All images are representative of multiple experiments.
Figure 3.3 - The novel splice variant of TMEFF2 produces a truncated protein. Immunoblot demonstrating expression of the GFP fusion protein constructs. Whole cell lysates from LNCaP cells not transfected or transfected with pRc-CMV, TMEFF2-GFP, or TMEFF2-S-GFP plasmids were immunoblotted using an anti-GFP antibody. Probing for β-actin confirmed the amount and quality of protein in each lane. Expression of TMEFF2-S produced a single 50kDa protein. Identical results were obtained in replicate experiments; a representative result is shown.
portions of the full-length protein in the plasma membrane [1,5]. This protein is also known to be glycosylated, thus altering its predicted migration pattern in acrylamide gels [1,5]. In contrast, the TMEFF2-S-GFP fusion protein produced a single molecular weight species of approximately 50 kDa, close to the predicted size of 48 kDa. A doublet band of about 27 kDa probably represents free GFP produced from internal ribosome entry into the fusion transcripts as this band was present regardless of the fusion protein being expressed. An unexpected band of 21 kDa was also seen. The identity of this band is unclear, but probably represents a cleavage product of the GFP protein as the antibody used was raised against the full-length GFP sequence. These results confirm that the TMEFF2-S transcript identified does encode a truncated protein.

3.3.3 The truncated isoform of TMEFF2 produced a secreted protein

The predicted protein sequence of TMEFF2-S lacks a transmembrane domain (Fig. 3.1C); this may alter its subcellular localization as compared with full length TMEFF2. To determine this, LNCaP cells transfected with the GFP fusion proteins were examined by fluorescence microscopy (Fig. 3.4A). As predicted, cells transfected with TMEFF2-GFP showed diffuse cytoplasmic localization with strong signal in the plasma membrane (Fig. 3.4A, top panels). In contrast, cells expressing the TMEFF2-S-GFP splice variant showed no signal in the plasma membrane (Fig. 3.4A, bottom panels). All TMEFF2-S-GFP fluorescence was observed in bright foci in the cytoplasm, again in contrast with the diffuse cytoplasmic signal seen with full-length TMEFF2. Thus, the subcellular localization of TMEFF2-S was distinct from TMEFF2.
untransfected
pRc-CMV
TMEFF2-GFP
TMEFF2-S-GFP
RPMI + 10% FBS

TMEFF2-S

TMEFF2

GFP

DAPI

Overlay
Figure 3.4 - The truncated variant of TMEFF2 is not localized in the plasma membrane but is secreted by transfected cells. (A) Fluorescent micrographs showing subcellular localization of GFP fusion proteins. LNCaP cells transfected with either TMEFF2-GFP (top row) or TMEFF2-S-GFP (bottom row) constructs were fixed and counterstained with DAPI prior to visualization by fluorescence microscopy. The left panel shows GFP signal, the middle panel shows DAPI-stained nuclei, and the right panel shows the overlay of these images. TMEFF2-S-GFP was not present in the plasma membrane, but instead all GFP signal was present in discreet foci in the cytoplasm. All images were captured at 630X magnification. (B) Immunoblot demonstrating the presence of the TMEFF2-S-GFP fusion protein in the media of transfected cells. LNCaP cells were not transfected or transfected with pRc-CMV, TMEFF2-GFP, or TMEFF2-S-GFP. All images are representative of multiple experiments.
Given that the truncated TMEFF2 protein still contained the putative signal sequence while lacking the transmembrane region, we proposed to determine whether the truncated TMEFF2 protein was secreted. Western blot analysis using anti-GFP antibody detected a distinct band of approximately 50 kDa only in the media of cells expressing the TMEFF2-S-GFP protein (Fig. 3.4B). The intensity of this band increased with increasing amounts of plasmid DNA transfected (Fig. 3.4B). The TMEFF2-S-GFP protein detected in the media migrated the same distance as TMEFF2-S-GFP in whole cell lysates. No GFP-reactive proteins were seen in the media of cells expressing full-length TMEFF2 or in culture media. The secretion of TMEFF2-S suggests this isoform may have a function distinct from that of TMEFF2.

3.4 Discussion

The expression of splice variants from a single genomic locus allows for greater diversity of gene products without the need for enlarging the entire genome. Alternative splicing events have been associated with a number of genetic diseases, including cancer [11]. Often, these splice variants alter the function of the other gene products expressed from that locus [12,13]. There are multiple examples of transmembrane proteins with splice variants lacking the transmembrane domain, resulting in a secreted isoform of the protein [13,14]. This is the first report to describe a secreted isoform of TMEFF2.

Full-length TMEFF2 is expressed predominately in the brain and in the prostate, with even stronger expression seen in prostate cancer [3,5,7]. In agreement with these data, expression of TMEFF2-S was detected in LNCaP prostate cancer cells, normal and cancerous human prostate tissue, and weakly in human testis tissue. Previous studies
have detected an unidentified minor band of approximately 3.5 kb when probing for TMEFF2 by Northern blot analysis [3,5,6]. The probes used in those experiments would overlap with the novel splice variant described here, and thus the 3.5 kb band observed may represent this truncated form of TMEFF2. Full-length TMEFF2 expression appears to be androgen-regulated, and also repressed by the c-Myc protein [5,6,15], but whether the truncated isoform is also regulated in this manner is not yet known. It will be of interest to assess whether the expression level of TMEFF2-S changes with the progression of prostate cancer, as this is seen for full-length TMEFF2 [6]. Intriguingly, the expression of the minor 3.5 kb band decreased after castration in the CWR22 model, but the kinetics of this decrease appeared different from that of full-length TMEFF2 [6]. The expression patterns of these isoforms in both normal and cancerous tissues needs to be assessed to help elucidate their potential roles in carcinogenesis.

The precise function(s) of TMEFF2 need to be examined further, as does the relationship of these isoforms. It will be interesting to determine if TMEFF2-S functionally interacts with TMEFF2 through co-operative signalling events or competitive binding. A thorough examination of the different binding partners of the various forms of TMEFF2 may help in this regard. Expression of full-length TMEFF2 in the prostate is restricted to the luminal epithelial cells [7,8]. Given the similarities in their expression profiles it is likely both proteins are expressed in this cell type, suggesting these isoforms may have roles in the normal secretory functions of the prostate. The extracellular domain of TMEFF2 can be cleaved, resulting in at least two different soluble proteins [1,5]. This cleaved form of TMEFF2 may be responsible for its functional effects and the soluble TMEFF2-S protein may then negatively regulate, or
synergize with, cleaved-TMEFF2. One form of cleaved-TMEFF2 produces a soluble protein with both follistatin-like (FS) domains intact, but without the EGF-like domain, suggesting these FS domains act in concert. Follistatin regulates signalling downstream of several members of the transforming growth factor-β (TGF-β) family of growth factor ligands. In contrast to full-length TMEFF2, TMEFF2-S contains only one FS domain. The absence of one FS domain may then enhance, or inhibit, the activity of this secreted isoform relative to the cleaved-TMEFF2 peptide. Additionally, the loss of the EGF-like domain implies TMEFF2-S would not induce phosphorylation of erbB4, in contrast to TMEFF2 [1]. The delineation of the functions of these isoforms will be made simpler once a reliable screen for TMEFF2 activity has been established.

Recently, two groups have published reports proposing the use of antibodies directed against TMEFF2 in the treatment of prostate cancer [7,8]. While both studies reported favourable results, the presence of a soluble form of TMEFF2 may not have been considered when these antibodies were designed. Clearly, further studies will be required to examine the circulating levels of TMEFF2-S in these patients and whether the secreted isoform may cross-react with the antibodies used. If so, the TMEFF2-S protein may decrease the efficacy of this approach by competitively binding the antibody away from the tumour cells. Alternatively, targeting of TMEFF2-S may provide added benefit to patients by altering the normal function of this novel isoform.
3.5 References


that is associated with disease progression and androgen independence. Int J Cancer 2001;94(2):178-184.


4 14-3-3 SIGMA INCREASES THE TRANSCRIPTIONAL ACTIVITY OF THE ANDROGEN RECEPTOR IN THE ABSENCE OF ANDROGENS*

4.1 Introduction

Prostate cancer is the second leading cause of cancer death among American men [1]. Organ-confined disease is treatable by means of radical prostatectomy or radiotherapy, but while initially effective, androgen deprivation therapy for metastatic disease is strictly palliative. The eventual emergence of androgen-independent disease leaves patients with few effective treatment options, none of which extend survival by more than a few months. The molecular mechanism of disease progression remains uncertain (see [2,3] for recent reviews).

The androgen receptor (AR) is a ligand-dependent transcription factor required for the regulation of androgen-dependent genes, such as prostate-specific antigen (PSA). Increased expression of PSA in the sera of men after androgen deprivation therapy is the hallmark for identifying androgen-independent disease. Numerous other androgen-regulated genes also become re-expressed with progression to androgen independence, indicating that the AR is active in these tissues [3,4].

Ligand-independent activation of the AR is suggested to be a significant contributor to this progression to androgen-independent disease [3,5]. One proposed mechanism by which the AR is activated in the absence of androgens is by the action of co-regulatory proteins stimulated by other signalling pathways [5,6]. For example, interleukin-6 (IL-6) caused ligand-independent activation of the AR through the activity of steroid receptor co-activator 1 (SRC1), the mitogen-activated protein kinase (MAPK)

* A version of this chapter is a manuscript in preparation. Quayle, SN, Sadar MD. 14-3-3 sigma increases the transcriptional activity of the androgen receptor in the absence of androgens. In preparation.
pathway, and signal transducer and activator of transcription 3 (STAT3) [7,8]. Increased expression and/or activity of these proteins has also been correlated with prostate cancer and the development of androgen independence [9-12]. Additionally, the co-activator p300 was recently suggested to activate the expression of PSA independent of the AR in LNCaP cells after long term treatment with IL-6 [13].

The 14-3-3 family of proteins regulate a large number of cellular processes, including apoptosis, response to DNA damage, and mitogenic signalling (for a review see [14]). Members of this family form homo- and heterodimers, which can then bind to a diverse array of target proteins. Binding of 14-3-3s to target proteins modulates the function of those targets by, for example, altering their subcellular localization or protein interaction partners. This family consists of 7 different isoforms that share a high degree of sequence identity and conservation [15]. Despite this conservation, numerous isoform-specific observations have been described, including differences in binding partners, subcellular localization, and structure [16-18]. 14-3-3 eta was previously shown to enhance AR transcriptional activity in the presence of ligand, with no effect seen in the absence of ligand [19]. Unfortunately, the effects of other members of this protein family were not reported. Therefore, we investigated the effect(s) of all seven members of the 14-3-3 family of proteins on the transcriptional activity of the AR in prostate cancer cells. We demonstrated that 14-3-3 sigma specifically increased the transcriptional activity of the AR, particularly in the absence of androgens. To our knowledge, this is the first report to examine the effects of all seven 14-3-3 isoforms in the same system under identical conditions.
4.2  Materials and methods

4.2.1  Cell culture

LNCaP cells obtained from Dr. L.W.K. Chung (Emory University School of Medicine, Atlanta, GA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/mL penicillin, and 100 μg/μL streptomycin. All chemicals were purchased from Sigma, unless stated otherwise. Androgen stimulation was performed by treatment of cells with the synthetic androgen R1881 (PerkinElmer, Woodbridge, Canada).

4.2.2  Plasmids

The ARR3-tk-luciferase reporter plasmid has been described previously [20]. PSA (6.1kb)-luciferase was a kind gift of Dr. J.-T. Hsieh (University of Southwestern Medical Center, Dallas, TX). The expression vectors for the seven isoforms of 14-3-3 were generously provided by Dr. H. Fu (Emory University School of Medicine, Atlanta, GA) [21]. The control expression vector pDEST26 was generated by removing the BsrGI fragment from the 14-3-3 zeta-encoding plasmid, eliminating the entire coding sequence. All plasmids were sequence verified to ensure they contained the entire known coding sequence of each 14-3-3 isoform.

4.2.3  Transfection and luciferase assay

LNCaP cells were plated at 3.0x10⁵ cells per well in 6-well plates. After 24 hr, transfections were performed in serum-free media using LIPOFECTIN® Reagent (Invitrogen) according to the manufacturer’s protocol. Each 14-3-3 expression vector
was transfected at 500 ng/well unless indicated otherwise, and each luciferase reporter construct was transfected at 1.0 μg/well. The total amount of transfected DNA was normalized to 3 μg/well by the addition of a promoterless plasmid [7,8]. After 24 hr serum-free RPMI 1640 containing R1881 (0.001 - 10 nM), or an equal amount of vehicle (ethanol), was added to the cells. Cell lysates were harvested after 24 or 48 hr incubation using Passive Lysis Buffer (Promega, Nepean, Canada). Luciferase activities were measured on a multiplate luminometer (EG & G Berthold, Germany) using the Dual Luciferase Assay System (Promega). Luciferase activities were normalized to the protein concentration of the lysates [7,22] as determined by Bradford assays [23]. All transfection experiments were performed in triplicate wells and repeated at least 3 times. The results are presented as fold induction relative to pDest26 alone.

4.2.4 AR siRNA

A 21-nucleotide double-stranded siRNA duplex targeting nucleotides 293-312 (5'-aagcccatcgtagaggccca-3') of the AR was obtained from Dharmacon Research (Boulder, CO). A control double-stranded siRNA (AR-SCR) targeting the inverse sequence of nucleotides 293-315 (5'-acccggagatgctacccgaa-3') of the AR was also generated. The efficacy of these molecules was shown previously [24]. LNCaP cells were plated at 2.0x10^5 cells per well in 6-well plates and incubated in RPMI 1640 with 5% FBS for 24 hr. Cells were fed fresh serum-free RPMI 1640 and transfected with 100 nM of siRNA duplex using OLIGOFECTAMINE® Reagent (Invitrogen) according to the manufacturer's protocol. After 24 hr the cells were incubated for a further 24 hr in the presence of 5% FBS prior to transfection with the luciferase reporters (1.0 μg/well) and
14-3-3 isoforms (1.0 μg/well) in serum-free media as described above. Again, the total amount of DNA transfected was normalized to 3 μg/well by the addition of a promoterless plasmid.

4.2.5 Western blot analysis

LNCaP cells were grown and treated in parallel with the siRNA-transfected cells analyzed for luciferase activity. These cells here harvested and washed twice in ice-cold PBS. The cell pellets were lysed in 2x SDS-PAGE loading buffer and boiled for five minutes prior to loading on 12% SDS-PAGE gels. Alternatively, cell lysates from the luciferase assays were also used for western blotting. The separated proteins were transferred to nitrocellulose membranes and blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for one hour at room temperature or at 4°C overnight, and then washed three times. The blots were probed with anti-AR (441)(1:500) or anti-His (H-15)(1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 to 2 hours at room temperature, washed three times, and incubated with the appropriate IR-labelled secondary antibody (1:5000, Molecular Probes) for 30 minutes at room temperature. All washes were performed with PBS containing 0.1% Tween20. Primary antibodies were diluted in Odyssey Blocking Buffer with 0.1% Tween20, while secondary antibody dilutions contained an additional 0.01% SDS. Visualization was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences).
4.2.6 Co-immunoprecipitation

LNCaP cells were seeded at 2.0x10^6 cells per 10 cm plate. After 24 hr, the cells were either mock transfected or transfected as above with the 14-3-3 sigma or pDest26 plasmids. The cells were incubated for a further 24 hr prior to treatment with 10 nM R1881 or an equal amount of vehicle (ethanol) as indicated. After 24 hr the cells were harvested and washed in Buffer 1 (40 mM Tris pH 7.4, 1x protease-inhibitor cocktail, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulphonylfluoride). The resulting cell pellet was freeze-thawed and re-suspended in modified RIPA buffer (20 mM Tris pH 7.4, 140 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 10% glycerol, 20 mM sodium fluoride, 1x protease inhibitor cocktail) by rotation at 4°C for 15 min. Approximately 10% of each input lysate was removed and the lysates were then precleared with 4 µg/mL agarose-conjugated anti-mouse immunoglobulin prior to immunoprecipitating the AR with 2 µg/mL anti-AR (441) antibody and 20 µL of beads conjugated with Protein A/G (Santa Cruz Biotechnology). The precipitated proteins were washed successively with modified RIPA buffer. The final pellet was then re-suspended in 2x SDS-PAGE loading buffer and western blots performed as above.

4.3 Results

4.3.1 14-3-3 sigma specifically enhanced AR transcriptional activity

To examine the effects of all the 14-3-3 isoforms on the transcriptional activity of the AR we utilized two different luciferase reporter constructs. The PSA (6.1 kb)-luciferase reporter construct contains both the enhancer and promoter regions of the
human PSA locus and is highly sensitive to androgens [25]. The second construct, ARR3-thymidine kinase (tk)-luciferase, is an artificial reporter containing three tandem repeats of the ARE1 and ARE2 regions of the rat probasin gene [20]. Both of these reporters were transiently transfected into LNCaP cells along with each isoform of 14-3-3, or the empty vector pDest26, and then treated with the synthetic androgen R1881 (10 nM) or its carrier, ethanol (0.00055 % v/v). Both reporter constructs displayed similar fold induction trends for each 14-3-3 isoform when compared to the empty vector (Fig. 4.1A,B). LNCaP cells treated with R1881 showed small inductions of reporter activity by the various isoforms of 14-3-3 relative to the empty vector (Fig. 4.1A). One-way analysis of variance (ANOVA) followed by a multiple comparison test determined that both the sigma (2.2-fold) and tau (2.0-fold) isoforms caused a statistically significant increase (p<0.01) in PSA (6.1)-luciferase reporter activity relative to the empty vector. Surprisingly, though, 14-3-3 sigma was also found to greatly increase the activity of both reporters in the absence of androgens (Fig. 4.1B; p<0.001). This effect was specific to the sigma isoform as no other isoform significantly increased the activity of either reporter construct in the absence of androgens. Western blotting with an anti-His antibody demonstrated that all the isoforms were expressed in these cells (Fig. 4.1C). Together, these data indicate that the 14-3-3 sigma protein is uniquely able to increase the transcriptional activity of the AR, especially in the absence of androgens.
Figure 4.1 - The transcriptional activity of the AR was activated specifically by 14-3-3 sigma. LNCaP cells were transfected with each 14-3-3 isoform or the empty vector pDest26 and either the PSA (6.1)-luciferase or the ARR3-tk-luciferase reporter construct, followed by treatment with either 10 nM R1881 (A) or vehicle (B). The results are presented as fold induction relative to empty vector alone, and the error bars represent the standard deviation of the mean of three independent experiments. *, p<0.001 as determined by one way ANOVA and Tukey's multiple comparison test. (C) Immunoblot demonstrating expression of each His-tagged 14-3-3 isoform in transfected cells. Probing for β-actin confirmed the amount and quality of protein in each lane. All transfections and treatments were performed in the absence of serum.
4.3.2 14-3-3 sigma enhanced AR activity in a dose-dependent manner in the absence of androgens

We next examined the effect of increasing amounts of 14-3-3 sigma expression on reporter activity. All levels of 14-3-3 sigma caused a small, but consistent increase in reporter activity when cells were treated with R1881 (Fig. 4.2A). In contrast, reporter activity increased in a dose-dependent manner when the cells were grown in the absence of androgens (Fig. 4.2B). This suggests that only small amounts of 14-3-3 sigma protein were sufficient to activate AR-dependent reporters, but in the absence of androgens increasing amounts of this isoform were able to further increase reporter activity.

4.3.3 14-3-3 sigma increased the sensitivity of AR to low levels of androgens

Since 14-3-3 sigma strongly activated these reporter constructs in the absence of androgens, we investigated the effect of the sigma isoform at the low levels of androgens observed in patients undergoing androgen deprivation therapy. To test this, a range of R1881 concentrations (0.001 - 10 nM) were used. While the absolute levels of luciferase activity increased with increasing R1881, the fold induction caused by 14-3-3 sigma was actually greatest at low concentrations of R1881 (Fig. 4.3). These results implicate a mechanism whereby 14-3-3 sigma is able to sensitize the AR to low levels of androgen.

4.3.4 Induction of PSA reporters by 14-3-3 sigma was dependent on AR

Finally, to determine whether the activation of these reporter constructs by 14-3-3 sigma was mediated through the AR, a siRNA oligonucleotide strategy targeting the AR was used to reduce the amount of AR protein prior to the expression of 14-3-3 sigma in
Figure 4.2 - Increasing amounts of 14-3-3 sigma increased the amount of AR activity in the absence of androgens. LNCaP cells were transfected with the indicated amounts of 14-3-3 sigma (σ) and the total amount of DNA transfected was kept equal with the addition of pDest26 empty vector. The cells were then treated with either 10 nM R1881 (A) or vehicle (B). The results are presented as fold induction relative to empty vector alone, and the error bars represent the standard deviation of the mean of three independent experiments. All transfections and treatments were performed in the absence of serum.
Figure 4.3 - 14-3-3 sigma sensitized the AR to low levels of androgen. LNCaP cells transfected with equal amounts of 14-3-3 sigma or pDest26 empty vector were treated for 48 hours with the indicated concentrations of R1881. The results are presented as fold induction relative to empty vector alone, and the error bars represent the standard deviation of the mean of three independent experiments. All transfections and treatments were performed in the absence of serum.
Figure 4.4 - Activation of the ARR3-tk-luciferase reporter construct by 14-3-3 sigma was dependent on the AR. LNCaP cells were transfected with an siRNA oligonucleotide targeting the AR (AR-siRNA) or a scrambled oligonucleotide (AR-SCR) prior to transfection with 14-3-3 sigma (σ) or pDest26 empty vector. The cells were then treated with 10 nM R1881 (A) or vehicle (B). Activity of the luciferase reporter is expressed as fold induction relative to empty vector plus AR-SCR oligonucleotide, and the error bars represent the standard deviation of the mean of three independent experiments. *p<0.02 as determined by Student’s t-test between cells treated with R1881 and vehicle in the presence of the AR-SCR control oligonucleotide. (C) Immunoblot demonstrating an efficient decrease in AR protein levels in the presence of the AR-siRNA oligonucleotide under both treatment conditions. Probing for β-actin confirmed the amount and quality of protein in each lane. All transfections and treatments were performed in the absence of serum.
these cells. Consistent with our previous results, cells transfected with a control scrambled oligonucleotide (AR-SCR) and 14-3-3 sigma showed a statistically significant increase in reporter activity in both the presence and absence of androgens (Fig. 4.4A,B; \( p<0.02 \)). Transfection with an AR-specific siRNA oligonucleotide (AR-siRNA) greatly reduced the expression of AR protein in these cells (Fig. 4.4C). As expected, knockdown of AR protein levels led to decreased reporter activity regardless of the presence of 14-3-3 sigma or treatment with androgens. Significantly, though, expression of 14-3-3 sigma in AR-siRNA-transfected cells did not result in increased reporter activity (Fig. 4.4A,B). These data suggest that the AR was required for 14-3-3 sigma to activate this reporter construct.

**4.3.5 14-3-3 sigma did not alter AR protein levels**

We next investigated possible mechanism(s) by which 14-3-3 sigma could increase AR transcriptional activity. We first sought to determine if 14-3-3 sigma could bind directly to the AR to alter its activity, as was previously observed for the glucocorticoid receptor (GR), a related steroid nuclear receptor [26]. However, we did not detect a direct interaction between these proteins by co-immunoprecipitation under the conditions tested (Fig. 4.5A). Samples of input lysates confirmed the expression of AR and 14-3-3 sigma in these cells. Immunoprecipitation enriched the relative amount of AR protein, but no 14-3-3 sigma was detected in these samples. 14-3-3 sigma was also previously shown to inhibit the transcriptional activity of GR by sequestering the receptor in the cytosol [26]. Therefore, we monitored the subcellular localization of a green fluorescent protein (GFP)-AR fusion protein in the presence and absence of 14-3-3
Figure 4.5 - 14-3-3 sigma did not interact with or alter the amount of AR protein in the cells. (A) LNCaP cells were mock transfected or transfected with 14-3-3 sigma (σ) or pDest26 empty vector prior to treatment with 10 nM R1881 or vehicle for 24 hours and immunoprecipitation with an anti-AR antibody. The lysates and immunoprecipitated samples were immunoblotted with antibodies specific for the AR and 14-3-3 sigma. (B) LNCaP cells were transfected with either 14-3-3 sigma, 14-3-3 epsilon (ε), or pDest26 and treated as indicated for 48 hours. All transfections and treatments were performed in the absence of serum. Whole cell lysates were harvested and immunoblotted with an antibody specific for the AR. Probing for β-actin confirmed the amount and quality of protein in each lane.
sigma. No significant difference in AR localization was observed in the presence or absence of androgens when compared to cells transfected with empty vector alone. Finally, it was recently shown that expression of 14-3-3 eta increased GR protein levels, by inhibiting degradation of the receptor, which lead to increased transcriptional activity of the GR [27]. However, western blot analysis demonstrated that the presence of 14-3-3 sigma did not alter the level of AR protein in these cells (Fig. 4.5B). Thus, while the AR must be present for 14-3-3 sigma to activate these reporter constructs, the expression of 14-3-3 sigma does not increase AR activity by simply increasing the amount of AR protein present in the cell or by significantly altering the subcellular localization of the AR.

4.4 Discussion

Members of the 14-3-3 protein family are involved in regulating a wide range of cellular events. Various isoforms of this family have also been previously shown to regulate the transcriptional activity of their target proteins, including steroid nuclear receptors [14,19,28]. However, the studies investigating the AR and GR have focused on individual isoforms and the experiments have been performed in a variety of cell types. This is the first report to examine the effects of all seven isoforms on the transcriptional activity of the AR in a single system. We have shown that 14-3-3 sigma, in contrast to the other family members, greatly enhanced the transcriptional activity of the AR in the absence of androgens.

In contrast to our findings with the AR, Kino, et al. [26] demonstrated that 14-3-3 sigma inhibited the transcriptional activity of the GR by sequestering the receptor in the
cytosol. Additionally, 14-3-3 eta was shown to enhance GR activity via two different mechanisms. This isoform was shown to alter the subcellular localization of the GR corepressor RIP140 and increase the amount of GR protein, with both mechanisms resulting in increased GR transcriptional activity [27,29]. Finally, 14-3-3 eta was also shown to increase the transcriptional activity of the AR, but only in the presence of androgens, and the mechanism by which this occurred was not examined [19]. Our results showed minimal induction of AR activity in the presence of 14-3-3 eta expression, but this difference in results is likely due to differences in the way these experiments were performed. Our experiments utilized the LNCaP cell line, which expresses an endogenous AR, and the PSA (6.1kb) and ARR3-thymidine kinase (tk)-luciferase reporters, which are well characterized androgen-regulated reporter constructs. In contrast, the experiments of Haendler et al. [19] were performed in PC3 prostate cancer cells stably transfected with the AR and employed a novel androgen-regulated reporter based on the CRISP-1 promoter.

Previous studies have focused mainly on activity of the receptor in the presence of ligand, and little to no increase in transcriptional activity was seen in the absence of ligand [19]. In contrast, our experiments highlight the unique ability of 14-3-3 sigma to enhance AR transcriptional activity in the absence of androgens. Consequently, our work suggests that 14-3-3 sigma may play an important role in sensitizing the AR to low levels of androgens. These findings may be most relevant in the setting of androgen independent prostate cancer. Androgen withdrawal therapy results in decreased, but detectable, levels of dihydrotestosterone in the prostate [30]; the expression of 14-3-3 sigma may enable the AR to still respond strongly to these castrate levels of androgen.
We investigated a number of mechanisms by which 14-3-3 sigma could activate the AR. Direct protein-protein interactions were previously demonstrated between 14-3-3 sigma and GR [26] as well as between 14-3-3 eta and both the GR and the AR [28,29]. However, we did not detect a direct interaction between the AR and 14-3-3 sigma in our co-immunoprecipitation experiments. Our results also suggest that expression of 14-3-3 sigma did not alter the amount or subcellular localization of AR protein. LNCaP cells do not express 14-3-3 sigma; therefore, the lack of difference was not due to endogenous levels of 14-3-3 sigma [31]. One potential mechanism yet to be investigated is whether 14-3-3 sigma may alter the amount of, or localization of, an AR co-repressor, leading to increased AR transcriptional activity. A comparable situation was observed for the GR where 14-3-3 eta was able to sequester the GR co-repressor RIP140 in the cytosol, thus allowing the GR to have greater activity in the nucleus [29]. While it would be interesting to determine what protein(s) 14-3-3 sigma is binding to mediate its effects on AR, 14-3-3 sigma has been demonstrated to interact with hundreds of proteins [32]. It is therefore difficult to speculate which of these interactions may be responsible for increasing AR activity. Alternatively, 14-3-3 sigma may influence AR activity indirectly, for instance by leading to altered phosphorylation of the AR or one of its co-regulators. It would be possible to assess the phosphorylation status of AR in the presence of 14-3-3 sigma using antibodies specific for each phosphorylation site, but unfortunately only a few such site-specific antibodies exist. Furthermore, much work remains to identify all AR phosphorylation sites activated \textit{in vivo} as well as the kinases and conditions responsible for these events [33].
14-3-3 sigma appears to have a number of characteristics unique from the other 14-3-3 isoforms. For example, 14-3-3 sigma tends to only form homodimers while other 14-3-3 isoforms readily form heterodimers with multiple other isoforms [18]. The determination of the crystal structure of 14-3-3 sigma revealed a structural basis for this finding [18,34]. Various other isoform-specific functions have also been described, for instance in the subcellular localization of the sigma and zeta isoforms [17]. Additionally, all other isoforms of this family were shown to bind the nuclear localization sequence of p27, but only 14-3-3 sigma was unable to bind this region [35]. Finally, Cdc25C was also shown to be bound by other 14-3-3 isoforms, but not sigma [16,36]. Structural analyses revealed a secondary surface on 14-3-3 sigma that influenced the specificity of its interacting partners [18]. This region contained three residues perfectly conserved in all isoforms except sigma. Mutation of these residues to the consensus sequence of the other isoforms enabled 14-3-3 sigma to interact with Cdc25C [18], revealing a potential mechanism by which 14-3-3 sigma is able to exert specific effects on target proteins. Thus, it is not unexpected that 14-3-3 sigma was uniquely able to alter the transcriptional activity of the AR in our experiments.

Loss of 14-3-3 sigma has recently been correlated with the development of prostatic intraepithelial neoplasia, and the majority of patients with invasive prostate cancer displayed low or no expression of this isoform [37-39]. This decreased expression is due to promoter hypermethylation in both primary prostate cancer samples and in the LNCaP cell line [31,38]. However, these studies also showed that a subset of patients appeared to retain 14-3-3 sigma expression. Significantly, Cheng et al. [37] also observed an increased frequency of expression of 14-3-3 sigma in the more poorly
differentiated samples of prostate cancer. Huang et al. [40] correlated increased nuclear localization of 14-3-3 sigma with decreasing differentiation of tumours. Consistent with this, strong expression of 14-3-3 sigma was observed in the androgen-independent prostate cancer cell lines PC3, DU145, and 22Rv1, and expression of 14-3-3 sigma has been correlated with increased chemoresistance [31,38,41]. Increased expression of 14-3-3 sigma was also observed in response to treatment with androgens, and a reporter containing the 14-3-3 sigma promoter was induced within three hours of androgen treatment [40]. Previous studies have suggested that genes regulated by the AR are re-expressed in androgen independent disease [4]. It will be interesting to examine the expression of 14-3-3 sigma in androgen-independent tissues as the presence of 14-3-3 sigma protein may lead to increased activation of the AR in a subset of patients with hormone-refractory prostate cancer.
4.5 References


22. Rowan BG, Weigel NL, O'Malley BW. Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation


38. Lodygin D, Diebold J, Hermeking H. Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. Oncogene 2004;23(56):9034-9041.


5 CONCLUDING CHAPTER

5.1 Expression profiling of novel clones

5.1.1 Custom cDNA microarray

Preliminary work has been performed to examine the expression profiles of the genes identified in the SSH experiments described in Chapter 2. Expression levels of a handful of these clones were examined using other techniques, such as northern blot analysis, but given the large number of candidate genes this was not an efficient method of validating the results of these experiments. To overcome this problem, we chose 156 of these clones and spotted them on glass slides as a custom cDNA microarray, allowing us to examine the expression level of all these genes in a single experiment. We also selected 57 genes whose expression was previously shown to change with disease progression. A number of positive and negative control genes were also selected to allow us to monitor hybridization efficiency and control for sample-to-sample variations.

Using the custom cDNA microarray I hoped to obtain data regarding a number of questions. First, I examined normal human tissue RNA samples to determine if any of the selected clones represented genes that were exclusively expressed in the prostate. Such genes could be important in the diagnosis and monitoring of prostate cancer. Second, I treated prostate cancer cells in tissue culture with synthetic androgens to identify novel androgen regulated genes. Third, I have screened a few samples from the LNCaP hollow fibre model to identify genes whose changes in expression consistently correlate with hormonal progression. These genes are the most likely to be involved in the process of hormonal progression and could thus represent diagnostic or therapeutic targets.
I also examined this data to try to identify a “signature” of gene expression levels representing different stages of hormonal progression. A number of studies have now been performed where large sets of clinical samples have been profiled using microarrays. These studies have found that distinct clinical subtypes of particular cancers, with different clinical outcomes, can be identified based strictly on the expression “signature” of the tumour [1-4]. Another study determined that the likelihood of metastasis could also be determined by examining the expression profile of the primary tumour [5]. While these findings are exciting, it should be noted that these gene expression signatures have not proven as reliable when used by other groups; a recent study has suggested that thousands of patient samples would be required to develop a truly robust expression signature [6]. While the preliminary analysis of the data from our custom cDNA array identified interesting trends, significant conclusions could not be drawn at this point as in each case more samples will be required in order to generate higher confidence in the data.

There were other difficulties that also limited the utility of these custom microarrays. For instance, the microarray approach was not sensitive enough to consistently detect some of the rare transcripts expressed at low levels. Since a number of the transcripts seemed to be at the limit of detection the reproducibility of the technique was diminished. To compensate for this I attempted to use greater sample volumes. While this may have increased the reproducibility, the increased sample demands limited the number of samples for which sufficient material was available. Finally, because of initial concerns about the sensitivity of the technique, only a few of the novel cDNA fragments were spotted on this version of the array. Thus, the major
limitation of microarrays – the ability to only detect those transcripts on the array – prevented us from examining the expression levels and changes of all the novel genes identified by SSH.

5.1.2 Low-density Taqman® array

As discussed in Chapter 1.5 there are a number of techniques available for monitoring expression profiles for multiple genes. There are also many techniques for examining the expression levels of individual transcripts. Northern blot analysis, for example, is a standard technique for characterizing expression levels and transcript size. However, the most sensitive technique is likely PCR. Unfortunately, standard PCR reactions are not quantitative and are thus not appropriate for determining expression level. On the other hand, real time quantitative PCR was developed specifically to allow for accurate quantitation of genomic DNA and transcript levels [7]. Briefly, in addition to a standard gene-specific primer pair, this TaqMan® approach also utilizes a gene-specific probe located between the two primers. This probe carries both a fluorescent molecule and a quencher, and the degradation of the probe via the 5’ nuclease activity of the polymerase results in the release of fluorescence at levels proportional to the amount of PCR product. Amplification of a specific product can then be followed in real-time using a specially equipped thermal cycler.

Applied Biosystems (Foster City, CA) has extended this technology with the development of the TaqMan® Low Density Array. This “array” consists of 384 unique real time PCR assays positioned at defined locations on a 384-well microfluidics card. Thus, this Low Density Array is analogous to a microarray in that pre-defined targets are
placed at discrete sites on the card. In contrast, though, this microfluidics card equally distributes a cDNA sample amongst the 384 wells, thus allowing for the expression monitoring of up to 384 genes in a single experiment. Additionally, real time PCR is extremely sensitive and highly reproducible [7].

Given the large number of potentially novel transcripts identified by SSH, we hoped to examine their expression levels in samples of prostate cancer. However, such experiments would be limited by the amount of sample available. A new study is currently being designed by Dr Sadar in which real time PCR assays would be designed for each novel transcript. These assays, as well as assays for genes previously associated with prostate cancer, such as PSA and AR, will be combined in a single TaqMan® Low Density Array. This array will then be screened with samples obtained via biopsy or radical prostatectomy from >50 patients with low grade prostate cancer who were treated with curative intent. At least 5 years of clinical follow-up data is also available for these patients. It is hoped that this method will identify which, if any, of the novel transcripts identified by SSH may be a useful marker for predicting clinical outcome after primary therapy. Again, there may not be enough samples to generate a highly reproducible gene expression signature, but it is hoped that the reproducibility of the TaqMan® technique will identify specific genes and gene signatures worthy of extensive follow-up.

5.2 Summary and future directions

The molecular mechanisms resulting in the development of HRPC are not completely understood. The AR appears to play a significant role in this process; however, it is not apparent how the AR is being activated in the absence of androgens.
Additionally, new markers of disease progression need to be identified to distinguish disease subtypes that are associated with different prognoses and therapeutic responses. In this thesis I have described the experiments I performed to identify and characterize genes associated with the hormonal progression of prostate cancer.

### 5.2.1 Novel transcripts

Suppression subtractive hybridization has been used in the past to study differential gene expression in the development and progression of prostate cancer [8,9]. However, few of these studies have examined changes in expression associated with the development of androgen independent disease, and fewer still have examined in vivo models of progression [9]. Additionally, these studies generally focused on transcripts derived from known genes. In contrast, our study sequenced all the cDNA fragments isolated from SSH in order to identify novel transcripts associated with hormonal progression in the LNCaP hollow fibre model. This model is unique in that it allows the in vivo study of hormonal progression with pure populations of prostate cancer cells. Aside from the known genes that were isolated, this SSH approach also identified sequences from 25 novel transcripts. These transcripts had not been previously detected and thus may represent novel genes of interest in prostate cancer. Additionally, a significant number of the isolated cDNA fragments corresponded to or overlapped with previously sequenced ESTs for which no other annotation was available. Such clones are informative for two reasons: (1) this is the first report to identify many of these ESTs in prostate cancer, and (2) some of the clones overlapping with known transcripts may
derive from novel splice variants of these genes. These studies have provided a rich source of novel transcripts in the hormonal progression of prostate cancer.

As described in Chapter 5.1, ongoing experiments are examining the expression levels of the transcripts identified by SSH. These experiments are aimed at (1) identifying transcripts whose expression is regulated by the AR, (2) identifying genes specifically expressed in prostate tissues and/or prostate cancer, and (3) identifying transcripts whose expression correlates with the development and progression of prostate cancer.

Aside from assessing expression changes in the LNCaP hollow fibre model, such experiments will also allow for the examination of other models of disease recurrence, such as the CWR22 xenograft model described in Chapter 1.4 [10,11]. Confirmation of the observed expression changes in multiple models of this process will increase confidence in their validity. The ultimate goal will be to validate these findings in clinical specimens of HRPC. However, such tissues are difficult to obtain, and thus, only the most promising candidate genes would be likely to progress to this stage. Additionally, other members of the Sadar Laboratory are examining expression changes of known genes in the LNCaP hollow fibre model using multiple high-throughput techniques such as Affymetrix GeneChip® arrays, SAGE, and Isotope-coded affinity tags (ICAT). It is hoped that these experiments will provide complementary data to aid in the selection of target genes for future experiments. Such an approach led to the selection of the 14-3-3 family for the functional studies described in Chapter 4.

As discussed in Chapter 1.1, new markers are needed to distinguish those patients likely to have a poor prognosis. The expression of such markers would ideally
be restricted to, or greatly upregulated in, prostate cancer cells. For example, PIM1 and hepsin were identified as potential biomarkers in prostate cancer through a microarray analysis [12]. Given their restricted expression profile, such markers are also potential targets of novel therapeutics. For example, both TMEFF2 (discussed in Chapter 3) and STEAP (six-transmembrane epithelial antigen of the prostate) were proposed as therapeutic targets after being identified as differentially expressed in prostate cancer through the use of subtractive hybridization [9,13]. Therapeutic antibodies targeting TMEFF2 have now shown favourable results in independent pre-clinical trials [14,15].

The potential utility of these novel transcripts in defining prognostic and diagnostic gene signatures should also be considered. Previous studies have used microarrays to identify gene expression signatures associated with prostate cancer recurrence after surgery and with the development of metastatic cancer [4,5]. But, as previously discussed, one of the great limitations of microarrays is that one can only detect transcripts that are represented on the array. Future studies defining the expression profiles of the novel transcripts described in Chapter 2 may thus identify novel biomarkers or expression profiles for later use in the clinical management of prostate cancer.

Finally, in order to begin functional studies on any of the identified transcripts it would first be necessary to isolate full-length cDNAs for these genes. Rapid amplification of cDNA ends (RACE) is a powerful technique to isolate full-length coding sequences, and it may prove to be more efficient when used on a gene-by-gene basis. Having this sequence information would allow for the prediction of protein coding
regions and possible functional domains, as well as comparison to previously identified genes in other species.

5.2.2 TMEFF2-S

The transmembrane protein TMEFF2 is primarily expressed in the brain and the prostate, with higher expression levels observed in prostate cancer [14,16,17]. As discussed in Chapter 3, one of the novel cDNAs isolated via SSH was shown to derive from a splice variant of the TMEFF2 gene. This truncated splice variant encodes a secreted form of the TMEFF2 protein that lacks an EGF-like and follistatin-like domain. This is the first report to identify a secreted isoform of the transmembrane protein TMEFF2. Further studies will have to be performed to determine the expression profile of the TMEFF2-S variant during the development and hormonal progression of prostate cancer. Being a secreted protein, it may also be possible to detect TMEFF2-S in the serum of patients. This is one of the ideal characteristics of a biomarker as it allows for easy, relatively non-invasive monitoring in patients.

These studies are timely and of high interest because Protein Design Labs, Inc. (Freemont, CA) and Berlex Biosciences (Richmond, CA) are developing antibodies to TMEFF2 for the potential treatment of metastatic prostate cancer based upon the limited tissue distribution of full-length TMEFF2 [14,15]. However, these groups may not have considered the presence of a secreted isoform of TMEFF2 when designing their antibodies. The potential mechanism(s) of action of these antibodies and the strategies used for epitope selection may have to be reconsidered in light of these results.
Additionally, this novel isoform of TMEFF2 may be a valuable reagent for examining the potential function and mechanism of the TMEFF2 protein in the etiology of prostate cancer and the development of androgen-independent disease. Having both isoforms would allow for the direct comparison and characterization of each form of the protein. For example, a thorough analysis of the binding partners of each isoform may delineate those protein-protein interactions which are isoform-specific. This knowledge may aid in the design of future experiments to examine the functional roles of each variant.

Since TMEFF2-S is a secreted factor it is easily amenable to studies of its effects on other cells. For instance, the culture media from cells expressing the TMEFF2-S-GFP fusion protein could be removed and applied to cells not expressing this construct. Comparison with media isolated from control cells would indicate if the presence of the secreted protein alters the growth characteristics or morphology of the treated cells. Alternatively, TMEFF2-S protein could be generated from an *in vitro* translation system or purified from a bacterial expression system. Given the EGF-like and follistatin-like domains it is possible that TMEFF2 and TMEFF2-S modulate the signalling events of a growth factor protein. Therefore, it may also be necessary to test the effect of TMEFF2-S before and after treatment with a variety of growth factors. The results of such experiments could be highly relevant to the understanding of HRPC as a number of growth factors have been implicated in the ligand-independent activation of the AR [18-20].

Finally, it is unknown whether TMEFF2 and TMEFF2-S would act at the level of the cell surface or in the extracellular milieu. For example, the latent TGF-β binding
proteins (LTBP) regulate the availability and activity of secreted TGF-β ligand [21]. Alternatively, a homolog of TMEFF2, called TMEFF1, acted at the level of the membrane to inhibit signalling downstream of the TGF-β family member Nodal. Nodal normally binds the type I and II receptors, but also requires binding to the co-receptor Cripto. TMEFF1 was shown to specifically inhibit signalling through Nodal at the level of the membrane by directly binding the Cripto co-receptor [22,23]. Only the transmembrane form of TMEFF1 exhibited this activity; a secreted TMEFF1 construct had no effect on Nodal signalling [22]. Once the binding partners of TMEFF2 are known it will be interesting to determine if a similar scheme is in place for TMEFF2. However, TMEFF1 is not currently known to encode a secreted isoform, thus it is difficult to predict what role TMEFF2-S may be playing.

5.2.3 14-3-3 sigma

When androgen-responsive prostate cells are maintained in the absence of androgens the majority of AR protein becomes localized in the cytoplasm, indicating it is transcriptionally inactive [24]. This is consistent with the low level of PSA expression observed in LNCaP cells maintained in androgen-free medium [25]. HRPC is characterized by the re-expression of PSA and other androgen-regulated genes, suggesting the AR is active in these tissues [26,27]. Much research effort is now focused on the mechanisms enabling the AR to be highly transcriptionally active in the presence of little to no ligand. The consistent identification of various members of the 14-3-3 family of proteins in high-throughput screens of differential expression in LNCaP cells led us to predict these proteins may be of interest in the development of HRPC. Chapter
described my experiments to show that 14-3-3 sigma may specifically sensitize the AR to low levels of androgens. This is the first report to demonstrate that a 14-3-3 family member could increase the transcriptional activity of a steroid nuclear receptor in the absence of its natural ligand.

A previous study showed that three amino acid residues in 14-3-3 sigma were critical for altering its binding specificity relative to the other 14-3-3 isoforms [28]. Given that only 14-3-3 sigma significantly altered AR activity in my study, it would be interesting to determine if mutating these three residues back to the consensus sequence would prevent 14-3-3 sigma from activating the AR. Such an experiment would confirm that a specific interaction with 14-3-3 sigma is required for this effect. However, it is difficult to predict which protein, or proteins, 14-3-3 sigma is interacting with in this situation. Work by Benzinger, et al., demonstrated that 14-3-3 sigma interacted with hundreds of different proteins under the conditions tested [29]. It is also likely that different cell types and treatment conditions would express a unique repertoire of binding partners. Nonetheless, it would be of interest to determine the protein-protein interactions mediating the effect of 14-3-3 sigma on AR. This could be done with a candidate-by-candidate approach, where known AR co-regulators, such as SRC-1 [30] and NCoR [31], could be tested for their ability to interact with 14-3-3 sigma.

One method to limit the possible list of candidate binding partners may be to assess the subcellular localization of 14-3-3 sigma in the presence and absence of androgens. This may establish in which cellular compartment the interaction is occurring. For instance, if the majority of cells show expression of 14-3-3 sigma only in the cytoplasm, this would suggest that most 14-3-3 sigma interactions would also be
occurring in the cytoplasm. In this case, it would be likely that 14-3-3 sigma was sequestering an AR co-repressor in the cytoplasm, thus allowing the AR to be more active in the nucleus. If, however, most 14-3-3 sigma was seen in the nucleus, it may suggest that 14-3-3 sigma is recruiting an AR co-activator to the nucleus, thus allowing the AR to be more active in the absence of androgens. The work of Huang, et al., showed that 14-3-3 sigma was mostly in the cytoplasm under androgen-free conditions in M12 prostate epithelial cells stably transfected with the AR [32]. Interestingly, treatment of these cells with either DHT or epidermal growth factor (EGF) resulted in the nuclear accumulation of 14-3-3 sigma [32]. Experiments need to be performed to determine if similar results are found in the LNCaP cell line, which expresses an endogenous AR.

This same study demonstrated that transcription from the 14-3-3 sigma promoter was activated by treatment with androgens, suggesting that 14-3-3 sigma may be an androgen-regulated gene [32]. Future studies should determine if the AR directly regulates 14-3-3 sigma transcription by examining whether AR can bind its promoter. Sequence analysis of the 14-3-3 sigma promoter could be performed to see if consensus AREs are present. Additionally, chromatin immunoprecipitation (ChIP) could be utilized to determine if the AR is able to directly or indirectly interact with regions of the 14-3-3 sigma promoter.

Several studies have shown that 14-3-3 sigma is downregulated with the development of prostate cancer [33-35]. However, both increased frequency of expression and increased nuclear localization of 14-3-3 sigma have been seen in more poorly differentiated tumours [32,33]. Studies examining the expression of 14-3-3 sigma need to be performed to determine its expression frequency in HRPC tissues. As
discussed in Chapter 1.4, there are multiple potential mechanisms leading to the survival and proliferation of prostate cancer cells in the absence of androgens. The ability of 14-3-3 sigma to increase the transcriptional activity of the AR in the absence of androgens indicates this may be a potential mechanism of ligand-independent activation of the AR in the subset of HRPC patients whose tumours express 14-3-3 sigma.

Finally, exogenous factors are also able to influence the regulation of the transcriptional activity of the AR. For example, Zhu, et al. [36] recently showed that tumour-associated macrophages secreted IL-1β. Signalling events downstream of IL-1β altered the recruitment of the NCoR co-repressor to AR transcriptional complexes, with the result that the antiandrogen bicalutamide now acted as an AR agonist [36]. Experiments such as these demonstrate that the control of AR activity is a tightly regulated process, and that numerous events can lead to alterations in the activity of AR. It will be interesting to examine the upstream mechanisms leading to the expression of 14-3-3 sigma and whether external factors can influence the ability of 14-3-3 sigma to increase the transcriptional activity of the AR.

5.3 Conclusions

In conclusion, the application of subtractive hybridization to the LNCaP hollow fibre model has identified numerous genes of interest in the hormonal progression of prostate cancer. The secreted isoform TMEFF2-S provides an excellent example of the capacity of subtractive hybridization to aid in the discovery of novel transcripts in such a model. Since it encodes a secreted protein, further examination of this splice variant may identify it as informative in predicting patient outcome. Furthermore, it will be
interesting to determine if the TMEFF2-S protein is bound by the therapeutic antibodies targeting TMEFF2. SSH, in conjunction with other high-throughput screening techniques, also led me to focus on the possible functions of the 14-3-3 family of proteins in advanced prostate cancer. The discovery that 14-3-3 sigma specifically increases the activity of the AR in the absence of androgens may be highly significant. Further studies will be required to determine if 14-3-3 sigma is active in a subset of HRPC patients. If so, its role in activating the AR may potentially be targeted for the development of novel therapeutics for the treatment of androgen-independent disease.
5.4 References


34. Lodygin D, Diebold J, Hermeking H. Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. Oncogene 2004;23(56):9034-9041.


6 APPENDIX

6.1 Animal care certificate

The University of British Columbia

Animal Care Certificate

Application Number: A05-1794
Investigator or Course Director: Marianne Sadar
Department: Medicine, Department of

Animals Approved: Mice Male athymic Nude mice, BALB/c Strain 180

Start Date: November 1, 2005 Approval Date: January 6, 2006

Funding Sources:

- Funding Agency: National Institutes of Health
  Funding Title: Genomic and proteomic analysis of androgen independent prostate cancer

- Funding Agency: Health Canada
  Funding Title: Proteomics associated with the progression of prostate cancer to androgen-independence.

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility
6.2 Biohazard approval certificate

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H03-0057

INVESTIGATOR OR COURSE DIRECTOR: Sadar, Marianne

DEPARTMENT: Medicine

PROJECT OR COURSE TITLE: Genomic and proteomic analysis of androgen independent prostate cancer

APPROVAL DATE: 06-01-04

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: National Institutes of Health

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

151